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CONTENTS

No. 1

No. 2

No. 3

No. 4

No. 5

No. 6

OBITUARY NOTICES.

EGERTON CHARLES GREY (1887–1928).

EGERTON CHARLES GREY was the second son of the late Colonel Arthur Grey. His earlier years were spent in Paris, after which he went to Australia and graduated in the University of Sydney. Here he carried out some experiments on the fatty acids of the human brain. In 1912 he came to England with an 1851 Exhibition Scholarship and commenced at the Lister Institute the work on the chemical action of bacteria which he continued to pursue until his death. He subsequently became a Beit Fellow and a John Foulerton Student of the Royal Society, working for some time at the Pasteur Institute, where he acquired a great admiration for the work of Pasteur, and at the Biochemical Laboratory, Cambridge. During this time he was awarded the degrees of M.A. (Cantab.) and D.Sc. (London) and completed the work for his medical qualification, which he had commenced in Sydney.

During the war he served as second lieutenant in the Royal Fusiliers, and was wounded at Gallipoli and invalided from the service. He subsequently entered the Navy and served as Surgeon-sub-lieutenant to H.M.S. *Nereide* and as interpreter in French and German, taking part in the operations in the Black Sea. After the war Grey was appointed Professor of Chemistry in the University of Cairo, and for his services in this capacity was decorated with the Order of the Nile. His experiences here led him to write a short book, *Practical Chemistry by Micro-Methods* (Cambridge and London, 1925), in which a method of teaching analytical chemistry with the aid of a minimum amount of apparatus and reagents is described.

In 1926 he relinquished his appointment at Cairo and undertook an investigation of the food problems of Japan on behalf of the League of Nations. He spent about six months in Japan and made, single-handed, analyses of all the chief foodstuffs of the country. The results form the subject of a report issued by the League of Nations and are also contained in a series of articles in the Japanese weekly review, *The Trans-Pacific* (July and August, 1927). He returned to work at Cambridge, where he died after a very short illness on August 10th, 1928.

Grey was a man of attractive but unconventional character, full of enthusiasm for his subject. His early death is a great loss both to his friends and to biochemistry.

Grey's experiments on the chemical action of *B. coli communis* commenced at a time when Penfold had just shown that by cultivation in a medium containing sodium chloroacetate, a strain of this organism could be isolated

which did not produce gas from glucose. The nature of the change thus produced in the enzymic armoury of the organism formed his first subject of investigation and led him to the idea that two (or more) independent sets of enzymes were active in this fermentation. He developed this idea by studying the progressive changes during fermentation. By using a large inoculation and by avoiding, as far as possible, the complications due to the growth of the organism he was able to distinguish three characteristic periods. Immediately following inoculation many of the organisms die and the products formed from glucose are mainly alcohol, acetic acid, succinic acid and formic acid. During the next period the production of these ceases, some of them are even decomposed, whilst part of the sugar is synthesised into a non-reducing carbohydrate and another part converted almost wholly into lactic acid. At the same time the surviving organisms begin to multiply. Finally the synthesised carbohydrate is hydrolysed and fermented along with the remaining sugar, both lactic acid and the alcohol group of products being formed. Another series of observations was made on the influence of aerobic or anaerobic cultivation on the subsequent behaviour of the organism to sugar. When the immediate past history of the organism has been anaerobic, the fermentation under anaerobic conditions yields very little or no lactic acid and much less succinic acid than when its conditions of cultivation have been aerobic. These observations led Grey to the conclusion, expressed in a paper which was in the Press at the time of his death, that *B. coli* can decompose sugar in at least two different ways, by a lactic fermentation and by a modified alcoholic fermentation. The latter of these is only possible when the organism has been recently grown in the presence of free oxygen. Generalising from this he concluded that alcoholic fermentation is due to the continued action under anaerobic conditions of an enzyme, zymase, which is the surviving portion of the respiratory mechanism of the organism. Whether or not these ideas are accepted, they bear witness to the breadth of vision which was characteristic of their author and provide ample ground for further investigation.

A. H.

DIARMID NOËL PATON (1859–1928).

The death of Diarmid Noël Paton in his 70th year on September 30th, 1928, on the day on which his resignation of the Regius Chair of Physiology in Glasgow University took effect, has removed one of the earliest research workers in the field of chemical physiology in this country. So long ago as 1884 he began an investigation on the subject of urea formation and to the end of his life he was keenly interested in all metabolic problems. His interests were not confined to an interpretation of chemical physiology in any narrow sense. He was an investigator in many other physiological fields. He worked on the endocrine glands long before they became a popular subject of research; he directed investigations into dietary problems, particularly of the poor, and, later, into the relation of nutrition to the incidence of rickets and of growth. His last work was on postural reflexes and postural apnoea in the duck and the swan.

He was a splendid teacher, expressing his views clearly and succinctly. Although he held strong opinions on many problems he was scrupulous to put before his students all aspects of the question. He demanded that his students should use their intelligences and make up their own minds. *Ex cathedra* utterances were anathema to him. He remembered, too, that the majority of his auditors were going to be ordinary practitioners of medicine. He was well equipped for stressing the practical aspect of physiology looking back, as he did, to his own early days when he was a Clinical Tutor at the Edinburgh Royal Infirmary. He regarded and taught physiology as a whole, not as a variegated pattern of specialist problems put together for narrow and self-contained specialist workers.

Although he did not suffer fools gladly, and although he was perhaps sometimes rather excessively intolerant, hidden within his rather formal but graceful exterior was a most warm and kindly heart. He was always ready to help to the full extent of his powers anyone in real distress or difficulty. He had a shy sensitive nature which yearned for friendship.

As a research worker Noël Paton was keen, energetic, critical and thorough. He hated all cheap and shoddy work and, above all, he loathed special pleading, specious reasoning and the suppression of data which did not fit the hypothesis put forward. He tried to play the game himself and he expected his antagonists to have his own high sense of honour. He lived for science; science was indeed a religion to him and he could not tolerate any desecration by insincere or half-hearted self-advertising work.

E. P. C.

I. THE CHEMICAL ACTION OF QUINONES ON PROTEINS AND AMINO-ACIDS. PART II.

By EVELYN ASHLEY COOPER and RAYMOND BENNETT HAINES.

From the Chemical Department, University of Birmingham.

(*Received November 15th, 1928.*)

It was concluded from previous experimental work [Cooper and Haines, 1928, 1] that the bactericidal action of the quinones was associated with their chemical action on the simpler cell-constituents, such as the amino-acids, rather than with their chemical reactivity with the complex colloids, *e.g.* proteins. Subsequently, however, it was found [Cooper and Haines, 1928, 2] that the temperature-coefficient for the reaction between benzoquinone and glycine was the normal one for a chemical process, yet quinone was twenty times more active as a germicide at 37° than at 20°. It was then found however that this considerable accession in bactericidal power with rise in temperature was not restricted to quinone, but was a common property of oxidising disinfectants. It would appear thus that the main factor in the germicidal action of the quinones is the interaction with the amino-acids, but that another factor fundamental in the action of oxidising disinfectants is operating simultaneously.

The earlier work had shown that there was a general correspondence in the relative germicidal power of benzoquinone and toluquinone and their chemical activity with various amino-acids. Benzoquinone was found to react from four to five times as rapidly with amino-acids as toluquinone, while the ratio of their bactericidal action varied on an average from five to seven (and sometimes higher). It was also found that the presence of salts increased the velocity of reaction with glycine. Further experiments have therefore been carried out to ascertain the relative effect of salts on reaction-velocity in the case of both benzo- and tolu-quinone, as, if it could be shown that the inorganic constituents of living cells increased the reactivity of benzoquinone more than that of toluquinone, some explanation of the foregoing discrepancy would be offered.

0·2 % solutions of the two quinones were prepared, (1) in water, and (2) in Ringer's solution (the bicarbonate being excluded). 0·5 g. of glycine was then dissolved in 100 cc. of each solution, and the amount remaining in solution at stated times was determined by withdrawing 5 cc. and titrating by Valeur's method (Table I).

Table I. % *remaining in solution at* 20°.

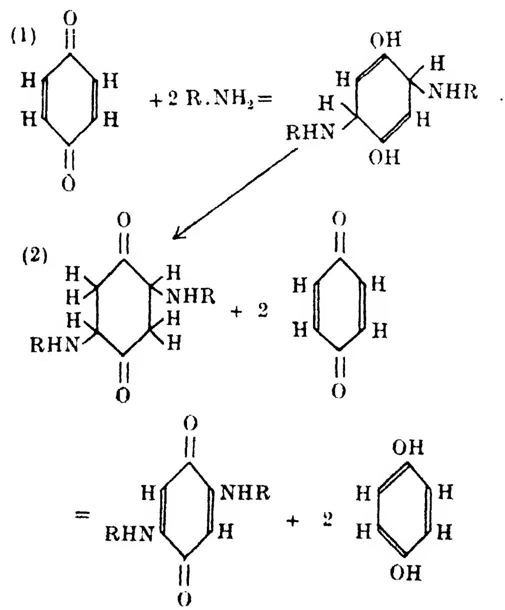
(*Benzoquinone.*)

Time min.)	(1) Water	Time (min.)	(2) Ringer's solution
0	100	0	100
10	98·7	10	93·7
30	96·6	35	90·8
70	90·4	100	86·0
120	87·2	160	81·2
235	83·5	240	74·9
360	74·5	360	65·2
	(*Toluquinone.*)		
0	100	0	100
20	98·2	10	99·5
60	96·4	35	96·4
120	95·2	60	94·8
180	92·3	165	92·1
240	91·1	240	90·5

The saline constituents of Ringer's solution thus considerably accelerate the reaction between benzoquinone and glycine, but have only a slight effect with toluquinone. The inorganic salts present in the cell may thus be supposed to raise the reaction-velocity of benzoquinone, and the great difference in the germicidal power of benzo- and tolu-quinone is explicable.

Apart, however, from velocity considerations, it will be shown that benzoquinone is capable of effecting more cell-destruction than toluquinone per gram-molecule.

Ville and Astre [1895] and Suchanek [1914] showed that the interaction of quinones and amino-compounds took place in several stages as follows:



The addition product can be isolated from either benzene or ether solution; in water or alcohol, however, the reaction continues by oxidation to the formation of the substituted quinone and quinol.

In the case of benzoquinone a dianilido-compound is formed, two nitrogen atoms being introduced. With toluquinone, although a similar addition compound is formed intermediately, the final derivative contains only one nitrogen atom. Thus three molecules of benzoquinone react with two of amino-compound, but two molecules of toluquinone react with only one of the amino-substance.

Xyloquinone forms an addition product, but is incapable of yielding a final substituted quinone, the reaction ceasing at the first stage.

Chloroquinones on the other hand [Astre, 1897] add on two molecules of amino-compound, and both nitrogen atoms finally enter the nucleus with evolution of hydrochloric acid. Chloroquinones are thus as effective as benzoquinone in taking the reaction completely to the oxidation stage.

These observations are analogous to those already made in the case of bactericidal action [Cooper and Haines, 1928, 1]. The homologues are less active as germicides than benzoquinone itself, whilst *m*-dichloroquinone is as efficacious as the latter.

Furthermore, alcohol facilitates the completion of the reaction to the oxidation stage, and also increases the germicidal power of the quinones.

The fact that three molecules of benzoquinone react with two of amino-acid, whilst two molecules of toluquinone are required for the reaction with one molecule of the amino-acid, may also explain the observation already referred to that the difference in the germicidal powers of benzo- and toluquinone is somewhat greater than would be expected from their reaction-velocities with amino-acids. It is evident that per gram-molecule benzoquinone would have a greater destructive effect on the cell than toluquinone in the proportion 3/2.

In order to throw further light on this problem the additive and final oxidation products were prepared from benzoquinone and anthranilic acid, and their germicidal action was studied. The additive product was only germicidal in a concentration as high as 1 in 3000 towards *B. coli* (30 min. at 20°); under the same conditions benzoquinone is active in 1 in 35,000. The oxidation product was too insoluble for examination. Although it would seem that it is the final oxidation process that plays the chief rôle in the bactericidal action of the quinones, the fact that benzoquinone suffers such an extensive depreciation in germicidal power merely by forming an addition compound with anthranilic acid suggests that the bactericidal process is also bound up to a certain extent with the preliminary addition reaction.

Reference has already been made to the finding that the temperature-coefficient for the interaction of benzoquinone and glycine is the normal one for a chemical process, whilst at 37° the quinone is twenty times as powerful a germicide as at 20°.

The exact germicidal temperature-coefficient, however, had not been determined under the conditions employed, all that had been ascertained being the relative concentration required for disinfection in a fixed period at 20° and 37°.

The temperature-coefficient for bactericidal action was therefore next measured by means of a modified technique.

Selected concentrations of phenol and quinone were inoculated with a definite volume of culture at 20° and 37°, and by subculturing at stated intervals into nutrient broth the periods required for complete disinfection at the different temperatures could be ascertained. The ratio of the times is a measure of the temperature-coefficient of the process (Table II).

Table II.

	Period of disinfection (min.)		Temperature-coefficient per 10°
	20°	37°	
B. coli			
Phenol, 1 in 200	150	27·5	3·2
Quinone, 1 in 50,000	72·5	7·5	5·7
B. fluorescens			
Phenol, 1 in 200	67·5	12·5	2·9
Quinone, 1 in 80,000	42·5	< 4	>6

The observations confirm the original suppositions; the temperature-coefficient of disinfection in the case of quinone is 6 or even higher, the coefficient for the chemical action on glycine being the normal one (2 for a 10° rise). In the case of phenol the temperature-coefficient for germicidal action is lower (approximately 3) and agrees more closely with the coefficient for the process of protein-coagulation by phenol, viz. 2 [Cooper and Haines, 1928, 2].

m-Cresol, on the other hand, was found not to increase in germicidal power with rise in temperature, 1 in 500 concentration being equally bactericidal at 20° or 37°. It was of interest therefore to extend the protein coagulation experiments to the case of this homologue of phenol.

100 cc. of egg-white were made up to 200 cc. with distilled water and filtered; the filtrate was diluted with an equal volume of water and again filtered. 100 cc. of the final filtrate were mixed with 100 cc. of 1 % *m*-cresol solution in a large bottle and kept at constant temperature in a thermostat. The rate of coagulation was determined at 20° and 30°, 10 cc. of the mixture being withdrawn at intervals and rapidly filtered.

The amount of protein in the filtrates was then estimated by heat-coagulation and weighing (Fig. 1).

The results, plotted in Fig. 1, show that the velocity of coagulation by *m*-cresol is only very slightly increased by rise in temperature, and are thus consistent with the bactericidal observations. The physico-chemical explanation of the germicidal power of the phenols is thus quite in accordance with the facts.

In the case of quinone, however, the chemical interpretation already put forward, whilst entirely consistent with many observations, does not at the present time account for the high temperature-coefficient for germicidal action.

Table III. % *quinone taken up from the solution.*

Pathological source of urine	Benzoquinone ½ hr.	Benzoquinone 1 hr.	Toluquinone ½ hr.	Toluquinone 1 hr.	Ratio ½ hr.	Ratio 1 hr.
Leucaemia	7·7	18·6	3·6	8·4	2·1	2·2
,,	67·6	—	37·9	—	1·8	—
Nephritis	11·2	25·0	8·2	18·1	1·4	1·4
Jaundice	5·44	25·0	5·2	25·1	1·0	1·0
Haematuria	41·0	66·0	28·6	53·1	1·4	1·3
,,	58·4	—	40·6	—	1·4	—
Glycosuria	35·8	—	6·1	—	5·9	—
,,	6·8	—	1·3	—	5·2	—

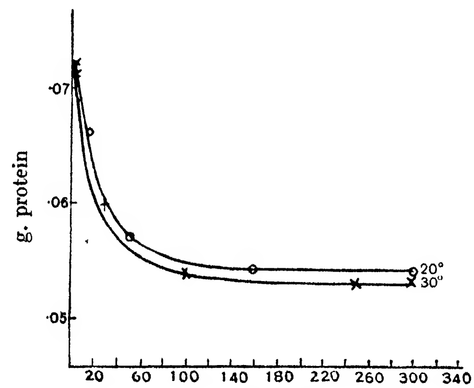


Fig. 1. Velocity of coagulation of albumin by *m*-cresol.

The possible application of the quinones in medicine.

It was previously shown [Cooper and Haines, 1928, 1] that alcoholic solutions of benzoquinone react rapidly with amino-acids, and since the nitrogenous constituents of urine vary considerably in health and disease it was thought that the amount of reaction might be of use as an index in diagnosis. Accordingly experimental work in this direction has been carried out. The clinical significance of the benzoquinone/toluquinone reactivity ratio has also been considered. In general, 10 cc. of the specimen of urine were allowed to react with an equal volume of 0·4 % solution of the quinones in 25 % alcohol for definite periods, the course of the reaction being followed by the volumetric estimation of the quinone remaining unused in the solution (Table III).

The chemical reactivity ratio of the two quinones is thus highest in the case of glycosuria (5·5), intermediate in value with leucaemia (2), and lowest in nephritis, jaundice and haematuria (1·4). It would thus seem that the deter-

mination of this ratio would afford a basis for a test which might be of value in medical diagnosis. The process resolves itself into a simple titration with thiosulphate under standard conditions, and can be carried out in 30 minutes to 1 hour.

The authors desire to express their best thanks to the Department of Scientific and Industrial Research for a maintenance grant which has made it possible to carry out this investigation.

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II. THE BACTERICIDAL ACTION OF THE NITROSO-COMPOUNDS.

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(*Received November 15th, 1928.*)

EARLIER investigations [Cooper, Woodhouse, and Forstner, 1925; Cooper and Forstner, 1926] have shown that the nitrosoanilines are much more effective germicides than the corresponding nitrosophenols. Nitrosoaniline, for example, is inhibitory in concentrations of 1 in 100,000, whilst with nitrosophenol concentrations of 1 in 20,000 are necessary to inhibit bacteria. Notwithstanding this activity it is characteristic of both types of compounds that their high germicidal power is only apparent in long period bactericidal tests, such as the inhibitory method which covers a period of 48 hours. In the ordinary short methods of measuring germicidal power ($\frac{1}{2}$–3 hours) the nitroso-compounds reveal only a low efficiency.

The present investigation has been concerned with the two following problems.

1. The possible application of the nitrosoanilines as internal disinfectants.
2. The mechanism of their bactericidal activity.

1. *The nitrosoanilines as disinfectants.*

Nitrosoaniline and nitrosodimethylaniline have the disadvantage of being slightly soluble in water. The hydrochloride is readily soluble, but is much less efficacious. The methyl iodide addition product, on the other hand, is not very soluble, and is also much less effective than the free base.

The results in Table I illustrate these points.

Table I. *Inhibitory concentrations.*

B. coli, 48 hours at 37°

Nitrosodimethylaniline,	1 in 170,000
Nitrosodimethylaniline hydrochloride,	1 in 7,500
Nitrosodimethylaniline methiodide,	$>$1 in 25,000

The loss in activity cannot be explained on the assumption of partition-changes between the phases within the cell caused by differences in solubility, as the readily soluble hydrochloride and the insoluble methiodide, which is, in fact, less soluble than the free base, are both weak germicides. It is concluded therefore that the fall in bactericidal power is associated with the change in

the valency of the amino-nitrogen to the quinquevalent state, and an essential condition for germicidal efficiency in the case of the nitrosoanilines is the presence of nitrogen in the tervalent state as in the amino- or substituted amino-group.

Nitrosodimethylaniline however is easily soluble in glycerol, propylamine, or formamide without significant loss in germicidal power, and the possibility of its clinical application is at present under investigation.

2. *The mechanism of bactericidal action.*

It has already been pointed out that the nitroso-compounds are slowly-acting germicides. In order to understand the nature of their bactericidal action, quantitative studies have been carried out to ascertain the reactivity of these substances towards amino-acids, proteins, lipins, and nucleic acids. These experiments involved the volumetric estimation of *p*-nitrosophenol and *p*-nitrosodimethylaniline in aqueous solution, and this was carried out by titration in acid solution with titanous chloride.

In general 30 cc. of the aqueous solution of the nitroso-compound was placed outside a viscose dialyser contained in a broad stoppered bottle. Inside the dialyser was placed 1 g. of the substance with which the solute was to react, *e.g.* protein, etc., either dissolved or suspended as the case might be in a measured volume of water. Equilibrium, *i.e.* maximum uptake of the nitroso-compound, was found in all cases to be complete within 72 hours. The initial and final concentrations being determined by analysis, the amount of nitroso-compound reacting or absorbed by 1 g. of the substance could be ascertained. The experiments were carried out at 20° (see Table II).

Table II. *Uptake of nitroso-compound* % (i.e. % *fall in concentration in aqueous phase*) *by* 1 *g.*

	(*a*) Gelatin	(*b*) Albumin	(*c*) Edestin	(*d*) Nucleic acid*
Nitrosophenol	4·9	3·5	4·2	9·7
Nitrosoaniline	5·0	–	0·0	46·0
Nitrosodimethylaniline	0·9	5·6	0·4	22·2

* The nucleic acid was first dissolved in dilute sodium hydroxide solution and the solution neutralised with acetic acid.

Consideration of the results shows that the nitroso-compounds are taken up in large amounts by nucleic acid, but only to a very small extent by proteins. Quantitative experiments showed that there was also no reaction at ordinary temperatures between nitroso-compounds and glycine, diamino-acids or a mixture of amino-acids from autolysed yeast. Furthermore, when solutions of nucleic acid and *p*-nitrosoaniline were mixed together and allowed to stand, a greenish black precipitate was gradually deposited, pointing to a chemical reaction between the two substances. The product was subjected to complete analysis with the following results:

$$C = 50{\cdot}8, \quad N = 14{\cdot}2, \quad H = 5{\cdot}6\ \%.$$

The nucleic acid from which the substance was prepared had the composition:

$$C = 32\cdot8, \quad N = 14\cdot16, \quad H = 5\cdot1\ \%.$$

It is difficult to draw definite conclusions, but it is possible that the compound is a salt of the nitroso-base with the nucleic acid. The reaction between nitroso-compounds and nucleic acid proceeds with extreme slowness and is scarcely perceptible in a period of three hours, but becomes measurable in periods of from 24 to 72 hours.

There is thus a distinct analogy in the case of nitroso-compounds between the process of germicidal action and the reactivity with nucleic acid, an essential constituent of cell nuclear material and associated with the mechanism of growth. Such cell-constituents as proteins and amino-acids are not appreciably attacked by nitroso-compounds, and the bactericidal action of these slowly acting disinfectants would appear to depend on a gradual but effective interaction with the nuclear constituents. Phenols such as phenol, the cresols, dihydroxybenzenes, and chlorophenols have no precipitating action on the nucleic acids, their germicidal action being more associated with their denaturing action on proteins.

With the object of throwing further light on the problem the influence of concentration on the uptake of *p*-nitrosodimethylaniline by nucleic acid was studied by the dialysis method already described (Table III).

Table III. *Final concentration of* p-*nitrosodimethylaniline.*

(*a*) Nucleic acid g. per g.	(*b*) Water-phase g. per cc.	Distribution ratio *a*/*b*
0·01139	0·000797	14·3
0·01577	0·001078	14·5
0·02330	0·001594	14·5
0·02612	0·001885	13·8

The distribution ratio tends to approximate to a partition-coefficient, the base being as much as 14 times as soluble in nucleic acid as in water. The constancy of this ratio suggests that a considerable proportion of the base is merely dissolved in the nucleic acid and the amount in actual chemical union is relatively small.

In order that the *p*-nitrosodimethylaniline may gain access to the nuclear substance of the cells, ready solubility in the intervening cell-constituents is essential, and a series of experiments was therefore next carried out to determine the solubility of the base in a lipin, *e.g.* lecithin.

A stable lecithin emulsion was prepared by dissolving the lipin in ether and pouring the solution into excess of water, the ether then being removed by means of a current of air.

A 5 % emulsion could thus be prepared. The influence of concentration on the absorption of nitroso-compounds, and also of quinones and phenol, was investigated, the dialysing method being again employed (Table IV).

Table IV. *Final concentrations.*

	(a) Lecithin g. per g.	(b) Water-phase g. per cc.	Distribution ratio a/b
Benzoquinone			
	0·01101	0·005119	2·2
	0·01061	0·004255	2·5
	0·00901	0·003386	2·7
	0·00793	0·002538	3·1
	0·00619	0·001684	3·7
Toluquinone			
	0·03060	0·005136	6·0
	0·02487	0·004303	5·8
	0·01592	0·003440	4·6
	0·01194	0·002720	4·4
	0·00448	0·001684	2·7
Nitrosophenol			
	0·04106	0·003599	11·4
	0·03367	0·003092	10·9
	0·02590	0·002546	10·1
	0·02122	0·001713	12·4
	0·01246	0·001182	10·5
	0·00646	0·000576	11·2
Nitrosodimethylaniline			
	0·01778	0·001301	13·7
	0·01119	0·000952	11·8
	0·00901	0·000796	11·3
	0·00659	0·000583	11·4
	0·00428	0·000398	10·8
	0·00165	0·000194	9·0
Phenol			
	0·5141	0·05501	9·3
	0·3758	0·04709	7·8
	0·1821	0·03558	5·1
	0·1741	0·02369	7·4
	0·0910	0·01184	7·7
	0·0396	0·00595	6·7

Inspection of the results indicates that the nitroso-compounds amongst the substances examined are most readily absorbed by the lecithin. In the case of *p*-nitrosophenol the distribution-ratio is almost constant over a wide range of concentration and the process is one of simple solution in the lipin, *p*-nitrosophenol being about 11 times as soluble in lecithin as in water.

The distribution-ratio of *p*-nitrosodimethylaniline increases somewhat with rise in concentration of 9·0–13·7, suggesting again a process of solution complicated by molecular association of the solute in the lipin. Similar results are obtained with phenol and toluquinone. With benzoquinone, however, the distribution-ratio falls with increasing concentration, suggesting that adsorption or surface-concentration predominates (Fig. 1). The constancy of the partition-coefficient in the case of nitrosophenol is of interest in view of the colloidal nature of the lipin phase.

Evidently the solubility of the substance in the lecithin is sufficiently high to obscure the surface-factor characteristic of colloidal systems. The results are analogous to those obtained in the case of the system water-phenol-protein [Cooper, *et al.*, 1912, 1923, 1927].

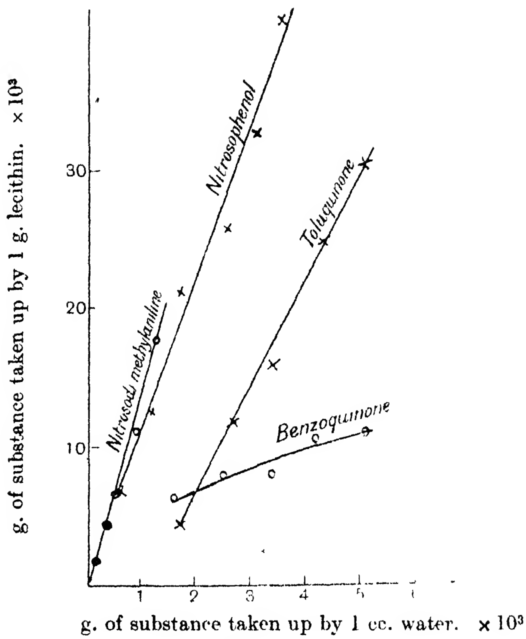
g. of substance taken up by 1 g. lecithin. × 10³
30
20
10
Nitrosophenol
Toluquinone
Nitrosodi methylaniline
Benzoquinone
1
2
3
4
5
g. of substance taken up by 1 cc. water. × 10³

Fig. 1.

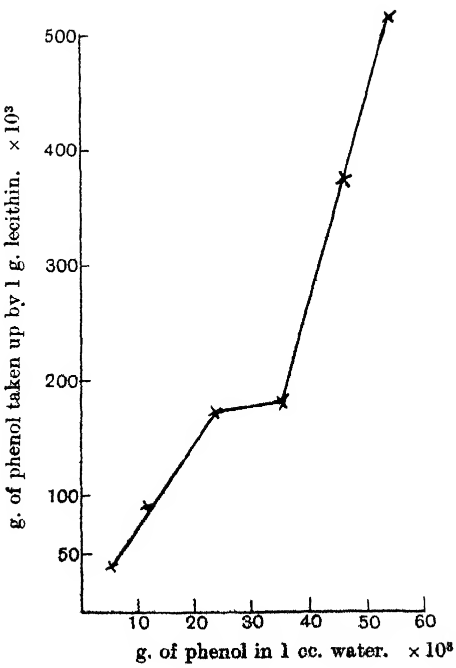
g. of phenol taken up by 1 g. lecithin. × 10³
500
400
300
200
100
50
10
20
30
40
50
60
g. of phenol in 1 cc. water. × 10³

Fig. 2.

The nitroso-compounds and quinones had no precipitating action on the lecithin, but with phenol precipitation of the lipin as a viscous mass occurred at a phenol concentration of 1–2 %, and it is at this point that a change in the distribution-ratio took place, as in the case of proteins (Fig. 2).

The results with *p*-nitrosodimethylaniline and toluquinone suggest that these compounds undergo molecular association when dissolved in lecithin, and in Table V the distribution-ratios obtained by dividing the square-root of the concentration in the lipin phase are tabulated.

Table V.

Concentration in Water phase (A) (g. per litre)	Concentration in Lipin phase (B) (g. per 1000 g.)	Square-root of $B = C$	Distribution-ratio C/A
Toluquinone			
5·136	30·60	5·532	1·07
4·303	24·87	4·990	1·16
3·440	15·92	3·987	1·16
2·720	11·94	3·450	1·26
1·684	4·48	2·012	1·19
p-Nitrosodimethylaniline			
1·301	17·78	4·219	3·24
0·952	11·19	3·347	3·51
0·796	9·01	3·000	3·89
0·583	6·59	2·567	4·40
0·398	4·28	2·069	5·20
0·194	1·65	1·285	6·63

In the case of toluquinone, the new distribution-ratio is almost constant over a wide range of concentration, showing that toluquinone is bimolecular when dissolved in lecithin. On the other hand, this constancy is not observed with *p*-nitrosodimethylaniline, the distribution-ratio being abnormally large at low concentration. It is probable that the nitroso-compound undergoes molecular association in lecithin, but is at the same time adsorbed to a certain extent, thus causing the distribution-ratio to vary.

Summary.

1. An essential condition for the bactericidal action of the nitrosoanilines is the maintenance of the amino-nitrogen in the tervalent state. The hydrochloride and methiodide are weak germicides.

2. The nitroso-compounds are slowly-acting disinfectants, exerting only a small bactericidal action in short periods, but this increases greatly in intensity in periods of 24–48 hours.

3. The nitroso-compounds have little or no action on amino-acids and proteins, but react gradually with nucleic acid forming a dark green insoluble product.

It is concluded therefore that the nitroso-compounds owe their slow germicidal action and marked inhibitory power to their gradual chemical interaction with the nuclear constituents of the cell, thus interfering with and retarding the biochemical mechanism of growth.

The authors desire to express their best thanks to the Department of Scientific and Industrial Research for a maintenance grant which has made it possible to carry out this investigation.

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III. STUDIES ON THE RELATIONSHIP BETWEEN CHEMICAL CONSTITUTION AND PHYSIOLOGICAL ACTION.

PART II. THE MIOTIC ACTIVITY OF URETHANES DERIVED FROM THE ISOMERIC HYDROXY-BENZYLDIMETHYLAMINES.

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(*Received November 27th, 1928.*)

In Part I of this series, the author [1926] synthesised, and tested for miotic activity, a number of substituted phenyl esters of substituted carbamic acids, thereby demonstrating that miotic activity was frequently associated with urethanes of this type. While certain variations could be made in the urethane grouping without necessarily destroying the activity of the compounds, greatest activity was observed with those urethanes which were esters of methylcarbamic acid. It was further found that both the nature and position of the basic group, which was present in all the compounds examined, could be varied without in all cases destroying the physiological properties of the urethane grouping. Nevertheless, an examination of the isomeric dimethyl-aminophenyl esters of methyl- and ethyl-carbamic acids showed that the power of these compounds to produce a contraction of the pupil varied according to the relative positions of the basic and urethane groupings, and, further, that conversion of the tertiary basic group into a quaternary ammonium grouping exerted a great influence on this power, in some cases increasing, and in others diminishing, the miotic activity. By application of the polarity theory the deduction was made that, for each series of isomeric urethanes examined, greatest activity was exhibited by those urethanes derived from the phenols with the smallest acidic properties, and the same conclusion can be reached by application of the more modern electronic theories.

In view of these results it appeared of interest to examine a new series of isomeric urethanes. The urethanes derived from the isomeric hydroxybenzyl-dimethylamines have therefore been prepared and tested for miotic activity. This particular series was chosen for two reasons. Firstly, it was desired to obtain substances the salts of which would not undergo appreciable hydrolytic dissociation in solution, as was the case when the basic group was attached directly to the benzene ring, since these would be more suitable for complete

pharmacological investigation. Secondly, it was hoped to gain further information regarding the relation between the miotic activity of the urethane and the acidic strength of the phenol from which it was derived.

The methods employed for the preparation of these compounds are described below and only one point requires comment. Whereas the dimethylaminophenols react smoothly with methyl *iso*cyanate to give the urethanes described in Part I, the reaction in the case of the hydroxybenzyldimethylamines is much more complicated. Using the conditions previously employed, the products from the *ortho*- and *para*-compounds appear to consist of a mixture of substances from which the required urethane can only be isolated with difficulty, if at all. By suitably modifying the conditions these difficulties have been overcome and, by strictly adhering to the conditions described in the experimental portion, the *N*-methylurethanes have been obtained in practically quantitative yield. In the case of the *N*-ethylurethanes, however, the difficulties have not been completely surmounted and these have only been isolated in the form of their salts.

Of the urethanes of this series which it has been possible to test, miotic activity has been confined to those which are esters of methylcarbamic acid; no activity has been observed with *N*-ethyl- or *N*-phenyl-urethanes. This corresponds with the results described in Part I, from which it was concluded that greatest activity was associated with esters of methylcarbamic acid. In conformity with the previous results, it has further been found that the activities of the three isomeric *N*-methylurethanes vary in intensity, the estimated order of the activities of the hydrochlorides being *ortho*- > *para*- > *meta*-. Not only does this order correspond with that of the lower homologues, but the difference between the activities of the *ortho*- and *para*-compounds has again proved to be much greater than that between the *para*- and *meta*-. Conversion of the tertiary basic group into the quaternary ammonium group intensifies the activity of the *ortho*-, diminishes that of the *meta*-, and abolishes that of the *para*-compound. This accords with the behaviour of the urethanes from the dimethylaminophenols in so far as the activities of the salts of the tertiary bases are different from those of the quaternary salts, but not as regards the direction in which the change occurs with the different isomerides. The author had hoped that it would have been possible to deduce the relative acidic strengths of the phenolic groups in the isomeric hydroxybenzyldimethylamines (and in their quaternary ammonium salts) from electronic theories, but unfortunately it appears impossible to do so in the present state of the development of these theories. Hence, while it is not improbable that the urethanes of these series which are the most active miotics are again those derived from the least acidic phenols, a discussion of this point must be deferred until it has been possible to measure directly the acidic strengths in question.

In Part I it was tacitly assumed that the miotic activity of the active urethanes was associated with the fact that they were phenyl, as distinguished

from alkyl, esters of carbamic acids, but no proof was advanced that similar activity might not be exhibited by urethanes derived from alcohols, provided these contained a basic group. The methylurethanes of two amino-alcohols, namely, dimethylaminoethyl alcohol and tropine, have accordingly been prepared. As anticipated, neither possessed miotic properties. Owing to the failure to obtain the hydrochloride of the first-named urethane in a crystalline condition the substance was tested in the form of its methiodide, which was therefore the methylurethane of choline iodide. It is interesting to note that the methylurethane of tropine exhibited neither miotic nor mydriatic properties. That it is not a mydriatic corresponds, of course, with the fact that the tropine is esterified with an aliphatic acid.

EXPERIMENTAL.

Urethanes derived from m-*hydroxybenzyldimethylamine.*

Methylurethane of m-*hydroxybenzyldimethylamine.* Finely powdered *m*-hydroxybenzyldimethylamine (1 g.), prepared according to the author's method [1927], was treated with excess of methyl *iso*cyanate. The phenol dissolved slowly with slight evolution of heat. The mixture was cooled in ice and, after 24 hours, light petroleum added. This caused the precipitation of only a small amount of oil. Hence all solvents were removed by distillation under diminished pressure, when the oily residue slowly crystallised. It was recrystallised by addition of light petroleum to its solution in benzene, when it formed thick plates which melted at 86°.

The *hydrochloride* separated from a solution of the base in alcoholic hydrochloric acid. Recrystallised from alcohol, it formed radiating clusters of prisms which melted at 175° with effervescence.

Analysis. 0·2448 g. gave 0·1423 g. AgCl.
9·06 mg. contained 1·05 mg. N (micro-Kjeldahl).
Cl found, 14·4 %; calc., 14·5 %.
N found, 11·6 %; calc., 11·5 %.

The *methiodide* was prepared by treating the base with excess methyl iodide and sufficient methyl alcohol to cause the solid to dissolve. After standing overnight the methiodide was precipitated by addition of dry ether. When recrystallised from alcohol it formed aggregates which melted at 162°.

Ethylurethane of m-*hydroxybenzyldimethylamine.* The finely powdered phenol was dissolved in ethyl *iso*cyanate and allowed to stand for 24 hours. Evaporation of the excess of ethyl *iso*cyanate under diminished pressure left an oil which could not be induced to crystallise. It was therefore divided into two portions, one of which was dissolved in alcoholic hydrochloric acid. After a short time the *hydrochloride* separated. It was recrystallised from alcohol when it formed rectangular tablets which melted at 156°.

Analysis. 0·2033 g. gave 0·1140 g. AgCl.
Cl found, 13·9 %; calc., 13·7 %.

The second portion of the crude base was dissolved in acetone and the solution treated with methyl iodide. The *methiodide* separated on standing. When recrystallised from alcohol it formed clusters of irregular plates which melted at 155–156° with effervescence.

Analysis. 0·1452 g. gave 0·0926 g. AgI.
I found, 34·5 %; calc., 34·9 %.

The results of a number of experiments made under varying conditions were practically identical with that of the above example. In no case was the base obtained in a solid condition, although the yield of salt was practically theoretical. Thus, in one experiment, 0·5 g. of *m*-hydroxybenzyldimethylamine yielded 1·1 g. of the methiodide of the ethylurethane.

Phenylurethane of m-*hydroxybenzyldimethylamine.* One molecular proportion (0·36 cc.) of phenyl *iso*cyanate was added to a cooled solution of 0·5 g. of the phenol in 5 cc. of benzene and the mixture left overnight in the refrigerator. The solution was then filtered to remove a small quantity of non-basic material which had separated. Evaporation of the solvents under diminished pressure yielded an oil which rapidly crystallised. When recrystallised from benzene, in which it is fairly soluble, it formed prisms which melted at 93°.

The *hydrochloride* separated when the base was dissolved in alcoholic hydrochloric acid. Recrystallised from alcohol it formed short prisms which melted at 175°.

Analysis. 0·2580 g. gave 0·1185 g. AgCl.
9·88 mg. contained 0·89 mg. N (micro-Kjeldahl).
Cl found, 11·4 %; calc., 11·6 %.
N found, 9·0 %; calc., 9·1 %.

Urethanes derived from p-*hydroxybenzyldimethylamine.*

Methylurethane of p-*hydroxybenzyldimethylamine.* One molecular proportion (0·24 cc.) of methyl *iso*cyanate was added to a cooled solution of 0·5 g. of the phenol in 5 cc. of benzene, causing the immediate formation of a slight precipitate. After standing overnight, light petroleum was added and a small amount of hygroscopic precipitate removed by filtration. The solvents were then evaporated at low temperature, when the residual oil crystallised. It was recrystallised by addition of light petroleum to a concentrated solution in benzene, when it formed a felted mass of fine crystals which melted at about 72°.

The *hydrochloride* separated when the base was dissolved in alcoholic hydrochloric acid. When recrystallised from alcohol it formed prisms with pyramid ends which melted at about 180° with effervescence.

Analysis. 0·2455 g. gave 0·1423 g. AgCl.
Cl found, 14·3 %; calc., 14·5 %.

The *methiodide* was prepared by addition of methyl iodide to a solution of the urethane in acetone. When recrystallised from alcohol it formed lustrous plates which melted at 188° with decomposition.

The *methochloride* was prepared by shaking for one hour a solution of the methiodide in methyl alcohol with a suspension of freshly precipitated silver chloride in methyl alcohol. The suspension was then filtered and the filtrate evaporated, when the residue, which consisted of the methochloride, crystallised. It was recrystallised from alcohol by the addition of dry ether, when it formed clusters of radiating needles which melted at about 110° with effervescence.

Analysis. 0·1575 g. gave 0·0855 g. AgCl.
Cl found, 13·4 %; calc., 13·7 %.

Ethylurethane of p-*hydroxybenzyldimethylamine.* The phenol (1 g.) was dissolved in dry acetone (10 cc.), and the cooled solution treated with one molecular proportion (0·6 cc.) of ethyl *iso*cyanate. The solvents were evaporated on the following day, when the oily residue partly crystallised. It was found impossible, however, to separate the crystals. The product was therefore dissolved in alcoholic hydrochloric acid. On addition of dry ether to this solution the *hydrochloride* separated as an oil which, after repeatedly washing with dry ether, mixtures of ether and acetone, and mixtures of ether and alcohol, finally crystallised. For complete purification it was necessary to recrystallise it three times by addition of ether to its alcoholic solution, when it formed rosettes of plates which melted at 165° with effervescence.

Analysis. 0·2105 g. gave 0·1148 g. AgCl.
Cl found, 13·5 %; calc., 13·7 %.

The same hydrochloride was obtained from the product of the action of an excess of ethyl *iso*cyanate on the phenol.

The *methiodide* was prepared by the addition of methyl iodide to a solution of the crude urethane in methyl alcohol, from which it was precipitated by the addition of dry ether. When recrystallised from alcohol it formed irregular plates which melted at 186°.

Analysis. 0·1787 g. gave 0·1170 g. AgI.
I found, 35·4 %; calc., 34·9 %.

Phenylurethane of p-*hydroxybenzyldimethylamine.* One molecular proportion (0·36 cc.) of phenyl *iso*cyanate was added to a cooled solution of 0·5 g. of the phenol in 5 cc. of benzene. The urethane quickly separated as an amorphous precipitate. This was filtered and crystallised from alcohol, when it formed thick prisms which melted at 126°.

The *hydrochloride* crystallised from alcohol in rosettes of needles which melted at 182°.

Analysis. 0·2455 g. gave 0·1132 g. AgCl.
12·72 and 14·19 mg. contained respectively 1·13 and 1·30 mg. N (micro-Kjeldahl).
Cl found, 11·4 %; calc., 11·6 %.
N found, 8·9 and 9·1 %; calc., 9·1 %.

Urethanes derived from o-*hydroxybenzyldimethylamine.*

Methylurethane of o-*hydroxybenzyldimethylamine.* The phenol (0·5 g.) was dissolved in 5 cc. of ether and a trace of metallic sodium added. This dissolved with the evolution of hydrogen. The solution was then cooled and one

molecular proportion (0·24 cc.) of methyl *iso*cyanate added. After some hours, a small quantity of flocculent material which had separated was removed by filtration and the filtrate evaporated at low temperature, when the residue crystallised. When recrystallised by addition of light petroleum to its solution in benzene it formed flat prisms which melted at 76°.

The *hydrochloride* was hygroscopic and could not be obtained crystalline.

The *methiodide* slowly separated from a solution of the base in acetone to which an excess of methyl iodide had been added. When recrystallised from alcohol it formed small prisms which melted at 175° with decomposition.

Analysis. 0·1565 g. gave 0 1045 g. AgI.
I found, 36·1 %; calc., 36·3 %.

Ethylurethane of o-*hydroxybenzyldimethylamine.* A number of experiments were carried out under different conditions, but in no case was it possible to isolate the urethane in a pure state. In the following experiment, however, a small quantity was obtained in the form of its methiodide. Attempts which have since been made to prepare larger quantities of this methiodide have unfortunately not been successful, although the experiments have been carried out under conditions as nearly identical as possible.

The phenol (0·5 g.) was dissolved in 5 cc. of dry ether and a trace of metallic sodium added. This dissolved with the evolution of hydrogen, when one molecular proportion (0·3 cc.) of ethyl *iso*cyanate was added. After standing overnight the solution was filtered from a small quantity of insoluble material and the filtrate evaporated. An oil was thus obtained which showed no signs of crystallising. It was therefore dissolved in acetone and excess of methyl iodide added. Addition of ether then precipitated an oily methiodide which, after standing till the next day, crystallised. It was filtered and the sticky substance thus obtained recrystallised from acetone, when it formed prisms which melted at 149° with effervescence.

Analysis. 16·16 and 14·49 mg. contained respectively 1·21 and 1·14 mg. N (micro-Kjeldahl).
N found, 7·5 and 7·8 %; calc., 7·7 %.

Phenylurethane of o-*hydroxybenzyldimethylamine.* A solution of 0·5 g. of the phenol in 5 cc. of benzene was treated with one molecular proportion (0·36 cc.) of phenyl *iso*cyanate. Some crystalline material separated slowly. This was filtered and washed with light petroleum (yield 0·48 g.). It was then warmed with a small volume of benzene, filtered from some insoluble crystals of a non-basic substance, concentrated, and placed in the refrigerator. A small quantity of the required urethane separated in the form of plates which melted at about 90°.

Analysis. 11·89 mg. contained 1·23 mg. N (micro-Kjeldahl).
N found, 10·4 %; calc., 10·4 %.

This urethane is apparently so unstable that considerable losses occur during recrystallisation.

The *methiodide* was prepared by warming the urethane with methyl iodide, dissolving the oil so produced in methyl alcohol, and then adding acetone and

dry ether, when the methiodide separated as a white solid. It was recrystallised by the slow addition of dry ether to its solution in acetone containing the minimum amount of methyl alcohol, when it formed rosettes of flat prisms which melted at 171°.

Urethanes derived from amino-alcohols.

Methylurethane of choline iodide. 1 g. of hydroxyethyldimethylamine, cooled in a freezing mixture, was treated with excess of methyl *iso*cyanate. After standing overnight, benzene and light petroleum were added, the mixture stirred, and the supernatant liquid decanted from a small amount of insoluble oil which was not further examined. The solvents were then evaporated, when a colourless liquid was obtained which would neither crystallise nor yield a solid hydrochloride. It was therefore dissolved in methyl iodide. A somewhat vigorous reaction took place and the methiodide separated in crystalline form. After addition of acetone, the crystals were filtered off and recrystallised from alcohol when they formed lustrous plates which melted at 174°.

Analysis. 0·1144 g. gave 0·0923 g. AgI.
I found, 43·6 %; calc., 44·1 %.

Methylurethane of tropine. The tropine, prepared from atropine, was dissolved in methyl *iso*cyanate and allowed to stand for some hours. The excess of methyl *iso*cyanate was then removed under diminished pressure and the residue crystallised from benzene, in which it is fairly soluble, when it formed rectangular plates which melted at about 126°.

The *hydrochloride* was precipitated as a sticky solid by addition of ether to a solution of the base in alcoholic hydrochloric acid. It was crystallised from a mixture of dry ether and alcohol, when it melted at 263° with decomposition.

Miotic activity of urethanes.

The tests for miotic activity were carried out on cats as described in Part I. In general, 2 % solutions of the urethane in Ringer's solution were employed. In the case of the methylurethane of *o*-hydroxybenzyldimethylamine, the hydrochloride of which has not been obtained in crystalline form, the base was dissolved in the calculated quantity of hydrochloric acid. The results obtained are given in the following table in which the estimated relative intensities of the activities of the active urethanes are indicated.

Substance	Miotic activity
m-$NH(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	+ +
m-$NH(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_3I$	+
m-$NH(C_2H_5).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	–
m-$NH(C_2H_5).CO.O.C_6H_4.CH_2.N(CH_3)_3I$	–
m-$NH(C_6H_5).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	–
p-$NH(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	+ + +
p-$NH(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_3I$	–
p-$NH(C_2H_5).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	–
o-$NH(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	+ + + +
o-$NH(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_3I$	+ + + + +
o-$NH(C_2H_5).CO.O.C_6H_4.CH_2.N(CH_3)_3I$	–
$NH(CH_3).CO.O.CH_2.CH_2.N(CH_3)_3I$	–

Summary.

1. The methyl-, ethyl- and phenyl-urethanes of the isomeric hydroxybenzyldimethylamines, and the methylurethanes of choline iodide and tropine have been prepared and, where possible, tested for miotic activity.

2. Miotic activity was confined to the methylurethanes of the isomeric hydroxybenzyldimethylamines, the urethanes of choline iodide and tropine being inactive in 2 % solutions. This confirms the view that activity is associated with the fact that the miotics are substituted phenyl, as distinguished from alkyl, esters of carbamic acids.

3. The activities of the methylurethanes of the isomeric hydroxybenzyldimethylamines are estimated to be in the order *ortho*- > *para*- > *meta*-. Conversion of the tertiary basic group into the quaternary ammonium group increased the activity of the *ortho*-compound, diminished that of the *meta*-, and abolished that of the *para*-.

The expenses of this investigation, which has been carried out during the tenure of a Carnegie Teaching Fellowship, has been met by grants from the Earl of Moray Research Fund of this University.

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IV. THE DENATURATION OF PROTEINS. PART V.

DENATURATION BY ACID.

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(*Received December 1st, 1928.*)

In earlier work carried out in this laboratory by P. S. Lewis [1926, 1927] on the heat denaturation of oxyhaemoglobin and egg-albumin it was shown that the unimolecular velocity constant characterising the process had a minimum value at the neutrality point of water (namely $p_H = 6{\cdot}76$ at 37°), and, further, that the process appeared to be catalysed approximately to the same extent by hydrogen ions and by hydroxyl ions. In the region of minimal velocity it was also found that the denaturation possesses an extremely high critical increment, namely of the order 77,000 cals. for oxyhaemoglobin, and 130,000 cals. for albumin. The temperature range for which these values were obtained was in round numbers 60° to 70°.

In view of these facts it was considered to be of importance to extend the p_H range as far as possible in the direction of increasing acidity, such extension necessarily involving the employment of lower ranges of temperature in order to deal with reaction velocities which would not be immeasurably rapid.

The present communication contains a summary of the results obtained. The proteins investigated, namely oxyhaemoglobin and albumin, were prepared and purified by the same methods as those employed by Lewis [1926, 1927]. Lewis's methods of determining the extent of denaturation at the various time intervals were also followed, with the exception that for denaturation at relatively low temperatures, such as 18°, it was necessary not only to cool the sample withdrawn, but also to adjust its p_H by addition of alkali to that of the isoelectric point to ensure that the denaturation itself is stopped, and also that all the protein denatured is precipitated.

The velocity of denaturation has been determined at 18°, 25°, 37° and 45°, over different ranges of p_H. The velocity constants given in the tables are in all cases calculated from the ordinary unimolecular expression in which the logarithm is to the base e and time is expressed in seconds. The stock solutions contained 0·5 % ammonium sulphate. It is known that this concentration of the salt does not appreciably affect the velocity of denaturation, and at the same time the ammonium sulphate not only acts as a preservative, but also ensures that the flocculation of the denatured protein shall be very rapid compared with the denaturation process itself.

All the results quoted are the mean values of concordant experiments. For the case of oxyhaemoglobin the average divergence of the mean values does not exceed 2 to 3 %; for albumin the error is somewhat larger. As a rule, the denaturation was followed to the extent of over 50 % in any individual run. The p_H was determined by means of the quinhydrone electrode at 25° in all cases.

Acid denaturation of oxyhaemoglobin.

Temperature 18°		Temperature 25°	
p_H of solution	$k_{uni} \times 10^4$	p_H of solution	$k_{uni} \times 10^4$
4·08	10·95	4·08	17·61
4·13	8·19	4·13	12·49
4·30	4·58	4·32	5·25
4·39	2·76	4·39	4·30
4·40	2·71	4·41	3·73
4·49	1·69	4·52	2·29
4·56	1·07	4·64	1·27
—	—	4·77	0·51

Temperature 37°		Temperature 45°	
p_H of solution	$k_{uni} \times 10^4$	p_H of solution	$k_{uni} \times 10^4$
4·11	30·65	—	—
4·15	22·71	4·23	25·30
4·31	12·26	4·45	11·10
4·48	6·31	4·52	7·26
4·51	5·15	4·63	4·36
4·60	3·22	4·74	2·74
4·68	2·19	4·90	1·21
4·85	0·80	5·10	0·59
		5·50	0·096

Acid denaturation of egg-albumin.

In connection with this protein previous workers have observed a change in p_H during the course of heat denaturation. Consequently in the present series of measurements several determinations of p_H values were made by means of the quinhydrone electrode at various points during the course of a run, and it can be quite definitely stated that, over the temperature range and p_H range investigated by the writer, there appeared to be no sensible alteration in p_H as a consequence of denaturation.

Temperature 25°		Temperature 37°	
p_H of solution	$k_{uni} \times 10^5$	p_H of solution	$k_{uni} \times 10^5$
1·02	1·59	1·02	30·0
1·17	1·35	1·32	8·83
1·35	0·82	1·46	7·00
1·52	0·64	1·58	5·56
1·72	0·48	1·62	5·68
		1·80	3·57
		1·99	2·16
		2·25	1·08
		2·55	0·91
		3·83	0·06

Temperature 45°

p_H of solution	$k_{uni} \times 10^4$	p_H of solution	$k_{uni} \times 10^4$
1·34	3·88	2·18	0·96
1·50	2·84	2·30	0·82
1·66	2·45	2·39	0·65
1·96	1·61	2·68	0·35
1·98	1·73	3·52	0·12

The critical increment.

This quantity, E, which represents the energy of activation of the denaturation process is calculated from the usual expression

$$\log \frac{k_2}{k_1} = \frac{E}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where k_2 and k_1 are the observed velocity constants at the two temperatures T_2 and T_1. The values of E corresponding to the temperature range and to a number of p_H values within the limits quoted above are recorded in the following tables. It may be pointed out that errors in the individual velocity constants become magnified in the calculation of the E values therefrom. The values quoted are consequently rounded.

Critical increment of the acid denaturation of oxyhaemoglobin.

p_H	Temp. range 18°–25° E in cals.	Temp. range 25°–37° E in cals.	Temp. range 37°–45° E in cals.
4·1	11,900	—	—
4·2	8,600	11,100	—
4·3	8,300	14,100	(6,600)
4·4	9,100	12,800	(4,000)
4·5	11,300	11,700	12,000
4·6	—	9,100	12,000
4·7	—	12,400	13,900
4·8	—	—	12,500

Critical increment of the acid denaturation of egg-albumin.

p_H	Temp. range 25°–37° E in cals.	p_H	Temp. range 37°–45° E in cals.
1·1	36,200	1·4	36,200
1·2	35,900	1·5	37,000
1·3	35,800	1·6	39,000
1·4	35,300	1·7	40,000
1·5	35,400	1·8	40,000
1·6	36,400	1·9	43,100
1·7	36,800	2·0	45,300
		2·1	48,100
		2·2	49,000
		2·3	48,200

Discussion.

Even when full allowance is made for inevitable experimental error the striking result, shown in the foregoing tables, is that in the region of moderate acidity the critical increment of denaturation, both for oxyhaemoglobin and for egg-albumin, is very much lower than it is in the neighbourhood of the

neutrality point of water in which region the process is known as heat denaturation. It is not proposed in the present paper to attempt to offer any explanation of this. It is not even known with certainty, as yet, whether or not the moderate values of E which hold for the low p_H range and relatively low temperature tend to merge gradually into the extremely high values of E obtained at the high p_H range and relatively high temperature characteristic of heat denaturation. If the change is a gradual one this would constitute an argument of some weight pointing to the fundamental identity of acid denaturation and heat denaturation respectively. Further work is being undertaken in this laboratory in this connection.

As we have just had occasion to raise the question of the relation of heat denaturation to denaturation by acid, it may not be out of place to draw attention to yet another mode whereby denaturation is said to be brought about, namely, mechanical denaturation. In this connection certain results obtained by Wu and Ling [1927] may be cited to the effect that, in contrast to other forms of coagulation which involve denaturation as a distinct stage, mechanical treatment (shaking) leads directly to the observation that maximum velocity occurs at the isoelectric point. Under ordinary conditions the speed of denaturation is slow compared with that of the (subsequent) flocculation of denatured material. As a consequence of mechanical treatment it would seem that the denaturation process has been greatly speeded up, so that the total observed effect is essentially a coagulation rate which would naturally be a maximum at the isoelectric point. So far as this evidence goes it affords some slight support to the view that denaturation is essentially a distortion or opening up of the protein unit, usually brought about as a consequence of localised chemical changes in the unit, but possibly much more rapidly brought about by mechanical treatment.

THE INFLUENCE OF FORMALDEHYDE UPON THE FLOCCULATION OF DENATURED PROTEIN.

Flocculation of a protein is the process which is subsequent to denaturation and is rendered possible by it. Flocculation is usually ascribed to the interaction of amino- and carboxyl groups situated on contiguous colloid units as a consequence of which these units increase in size by aggregation and eventually are coagulated. If this view be correct, then, if by any means either of these groups be blocked or inactivated, the resulting protein should not exhibit flocculation. To this end formaldehyde at various concentrations was caused to react with protein and the resulting material examined at various p_H values including the isoelectric point itself.

According to Harris [1923] the simplest mode of reaction of formaldehyde with an amino-group will lead to the formation of a methylene-imino-linkage, such reaction, however, not going to completion but attaining an equilibrium.

The results obtained, probably owing to the existence of the equilibrium

just referred to, were not as conclusive as had been hoped. They may be very briefly summarised as follows.

(*a*) In solutions of denatured oxyhaemoglobin containing formaldehyde (up to 0·5 %), some precipitation occurs on the acid side of the isoelectric point as far as p_H 3, and at the isoelectric point. There is no precipitation at p_H 7 and at higher p_H values.

(*b*) In solutions of denatured oxyhaemoglobin not containing formaldehyde there is complete precipitation at the isoelectric point and within a certain range on either side thereof, *i.e.* precipitation is still practically complete even at p_H 8 and decreases as the solution becomes more alkaline, whilst on the acid side the precipitate is still fairly complete even as far as p_H 3. The range over which precipitation occurs is greater in the absence of formaldehyde than in its presence.

As in the case of denatured oxyhaemoglobin the p_H range and degree of precipitation of denatured egg-albumin are both decreased by the addition of formaldehyde, and the greater the amount of formaldehyde added the greater the decrease.

The behaviour of both proteins towards formaldehyde may therefore be taken as evidence in support of the view that flocculation involves the amino- (and consequently the carboxyl) groups.

Summary.

1. The unimolecular velocity constants of the denaturation, by means of acid, of the two proteins oxyhaemoglobin and egg-albumin, have been determined at various temperatures and over a considerable p_H range.

2. From the observed velocity constants the corresponding values of the critical increments of activation for different temperature ranges have been calculated. It is found that for the acid denaturation of oxyhaemoglobin the critical increment has an average value of 12,000 cals. For egg-albumin the corresponding quantity lies between 36,000 and 48,000 cals.

3. Attention is drawn to the marked difference in the magnitude of the critical increments recorded and those already obtained for so-called heat denaturation, namely, 77,000 cals. for oxyhaemoglobin and 130,000 cals. for egg-albumin. Whether the values for acid denaturation merge into those for heat denaturation as the acidity and temperature range are altered to those usually associated with heat denaturation is under investigation.

4. The influence of formaldehyde upon the flocculation of denatured protein has been examined. It is found that the p_H range over which precipitation occurs is greater in the absence of formaldehyde than in its presence. This serves to confirm the generally accepted view that flocculation involves the amino- (and consequently the carboxyl) groups.

This investigation was carried out under the direction of Professor W. C. M. Lewis, F.R.S. The author desires to acknowledge his indebtedness to Imperial Chemical Industries Ltd., for a grant to the Department of Physical Chemistry of the University of Liverpool which partly covered the expenses of the work.

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V. THE DETERMINATION OF SMALL QUANTITIES OF STARCH IN VEGETABLE TISSUE.

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(*Received December 5th, 1928.*)

In some work on the reserve foods of the bracken fern, it was found necessary to use an accurate method of estimating starch in small quantities. Methods commonly in use which involve comparison of determinations of the sugar mixture, obtained after hydrolysis, by reduction of copper salts, and by the polarimeter, were not suitable as the quantities were too small for the use of the polarimeter. A method was therefore devised by which the copper-reducing power of the hydrolysed starch in the plant material was related to the reducing power of known quantities of a carefully purified starch preparation. Such a method does not give absolute values as the reducing power of starches of different origins is not the same. It does, however, give values comparable with each other for any one material and this is sufficient for many purposes, such as the comparison of the amounts of starch present at different stages of development.

The standard starch used for comparisons was prepared as follows. Lintner's soluble starch was washed carefully by decantation with six changes of distilled water. The remaining water was removed by washing in a similar way with three changes of absolute alcohol and drying to constant weight at 105°. Amounts of this preparation ranging from 0·1 to 0·8 g. were then weighed into clean dry conical flasks of 125 cc. capacity and 40 cc. of distilled water added. The mixture was then boiled for 30 seconds to emulsify the starch and cooled to room temperature. 5 cc. of a 0·2 % or 0·4 % aqueous solution of takadiastase (Parke Davis and Co., Ltd.) were added together with 0·05 cc. 5 % acetic acid and 0·2 cc. toluene, and the mixture was incubated at 36·8° for 24- or 48-hour periods. The same concentration of enzyme was used throughout; 0·2 % or 0·4 % gave very nearly the same results but smaller concentrations led to variations. Trials showed that after 48 hours the reaction was not quite complete, though even after 24 hours it was sufficiently slow to give consistent results, while shorter periods gave variable results.

The most suitable temperature was found by experiment to be 37°. At lower temperatures hydrolysis was slower and the copper-reducing power was not consistent. The temperature of the incubator was regulated to ± 0·3° about a mean of 36·8°, whilst that of the hydrolysis liquid varied ± 0·1° about the same mean. The addition of the acetic acid fixed the p_H at 5·5, at which value it remained constant throughout the reaction.

The copper reducing power was determined by Shaffer and Hartmann's method [1921] after several others, such as those of Pavy, Bertrand and Benedict, had been tried. All determinations were carried out in the following manner. The hydrolysis mixture, after the proteins, tannins and mucilages had been precipitated (see later), was neutralised to litmus and made up to a definite volume. 50 cc. of this solution were then pipetted into a conical 750 cc. flask and 50 cc. of Shaffer and Hartmann's reagent added, together with some pieces of unglazed plate. The mixture was brought to boiling point in four minutes, boiled for exactly five minutes, and cooled for three minutes under running water. As a constant source of heat an electric hot plate or a Bunsen burner, shielded from air currents by enclosure in a suitably ventilated tin, was used. A reflux condenser prevented concentration of the liquid by evaporation. The "cuprous titration" [Shaffer and Hartmann, 1921] was used exclusively.

A series of determinations upon different quantities of prepared starch was thus made. For use, it is convenient to plot the copper values (mg. copper reduced) against the weight of starch employed. The points fall very accurately in a straight line and from this graph, the amount of starch in the sample of plant material corresponding to the amount of copper reduced may be readily obtained. As has been said, this is only relative.

Table I. *Standard starch/copper values after hydrolysis with 0·2 % takadiastase.*

24 hours' incubation		48 hours' incubation	
Wt. of starch (mg.)	Wt. of copper reduced (mg.)	Wt. of starch (mg.)	Wt. of copper reduced (mg.)
31·0	47·4	20·0	36·1
40·0	56·2	30·0	50·0
53·2	67·4	41·0	62·4
60·0	76·8	59·8	82·4
71·4	86·5	69·7	92·9
89·9	106·9	78·5	105·6
100·1	116·5	80·0	108·4
110·0	123·0	88·1	115·9
119·9	135·3	103·5	129·7
140·0	157·9	108·7	134·7
—	—	119·9	152·4
—	—	139·9	171·0

The plant material, in this case bracken rhizome, was washed in water, killed by immersion in a bath of boiling methylated spirit to which sufficient ammonia (sp. gr. 0·8) had been added to make the total concentration of ammonia 1 %, and dried for 24 hours at 105°. The weight was then constant, as far as constant weight is possible with hygroscopic material. The dried material was powdered and could then be stored in well-stoppered bottles without showing any fungus growth even after 3 years.

A weighed amount of the dried tissue powder was placed in a tared Soxhlet extraction thimble and extracted for 24 hours with absolute alcohol in a Soxhlet apparatus, dried for 12 hours at 105° and weighed again. It was

absolutely necessary to conduct these weighings in a stoppered weighing-bottle. This extraction removed the sugars, chlorophyll and some of the proteins, and also rendered the material more easily soakable with water afterwards. 4 to 10 g. of the dry powder were weighed into a dry conical 125 cc. flask, emulsified with 40 cc. of water and hydrolysed in exactly the same manner as the standard starch. The reaction was stopped at the end of 24 or 48 hours by the addition of the reagent used for precipitating the remaining proteins, tannins, and mucilages.

Two such reagents were used with equal success and equally without effect upon the copper-reducing power of the products of hydrolysis.

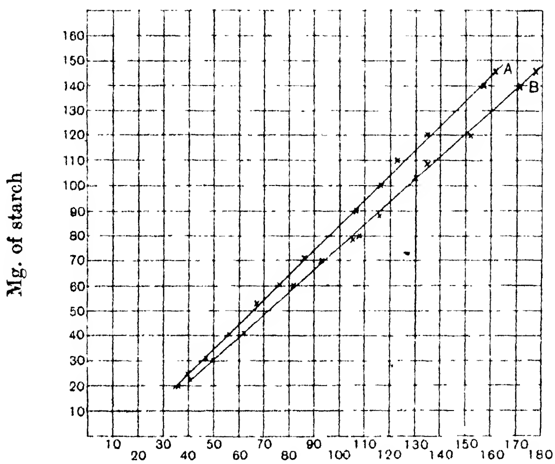

Fig. 1.

Standard starch/copper values

A. 24-hour period incubation.

B. 48-hour period incubation.

(1) *Lead acetate.* 20 cc. of a saturated solution of neutral lead acetate (A.R.) were added and the lead was almost entirely precipitated by the addition of 20 % potassium hydroxide, excess of the latter being avoided to prevent re-solution of any protein material. Upon filtration, a practically colourless liquid was obtained which contained no tannin, mucilage or protein. The excess of lead was removed by passing hydrogen sulphide, filtering, and subsequently aerating the filtrate for ten minutes to remove excess of hydrogen sulphide. The liquid was neutralised to litmus by potassium hydroxide and made up to a definite volume.

(2) *Mercury salts.* This was a modification of the method described by Thomas and Dutcher [1924], the mercury solution being prepared according to their formula. 20 cc. of this solution were added to the hydrolysis liquid after incubation. Solid sodium bicarbonate was then added in small quantities until frothing ceased, followed by 5 % sodium bicarbonate solution until

neutral to litmus. After filtration to remove the precipitate, which included the tannins, proteins and mucilages, the filtrate was acidified with dilute hydrochloric acid. The remaining mercury was precipitated by passing hydrogen sulphide and filtering. Excess of hydrogen sulphide was removed by aeration as before, and the liquid neutralised to litmus. The copper-reducing power was then determined in the same way as for the standard starch.

Successive estimations on the same sample of rhizome gave concordant results, *e.g.*

	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)
	16·0	26·4	14·2
	16·2	26·0	14·4
	16·5	26·6	14·6
Average	16·2	26·3	14·4

A further test of the reliability of the method was given by estimating the amount of starch in a rhizome sample, adding a known weight of the prepared starch to another portion of the same material and making an estimation of the total starch.

The results of two such experiments show a very close agreement.

Wt. of starch in dried rhizome (g.)	Wt. of starch added (g.)	Wt. of starch determined (g.)
0·712	0·105	0·820
0·630	0·050	0·690

Summary.

This paper describes a method for comparative estimation of small amounts of starch in vegetable tissue.

I am indebted to Professor O. V. Darbishire, the late Mr C. Hunter, M.Sc., and to Dr Macgregor Skene, for many helpful suggestions.

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VI. NOTE ON THE CHARACTERISATION OF THE ANTHOCYANINS AND ANTHOCYANIDINS BY MEANS OF THEIR COLOUR REACTIONS IN ALKALINE SOLUTIONS.

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(*Received December 11th, 1928.*)

Fear and Nierenstein [1928] have pointed out that the colour reactions of the anthocyanins, which are indicators, should be examined in solutions of definite p_H and their contention is undoubtedly correct. It would be a mistake, however, to conclude that the earlier observations are entirely lacking in significance simply because this refinement has not hitherto been adopted, and, for example, the effect of elimination or modification of the hydroxyl in position 3 in the pyrylium ring on the colour reaction is very marked. Thus both luteolinidin chloride [Pratt and Robinson, 1925] and chrysanthemin chloride [Willstätter and Bolton, 1916] give violet solutions in aqueous sodium carbonate and these become blue on the addition of sodium hydroxide; cyanin chloride [Willstätter and Everest, 1913] gives a blue solution in aqueous sodium carbonate.

It was inferred that chrysanthemin chloride should be the 3-glucoside of cyanidin chloride and this has since been proved to be correct by the synthesis of the anthocyanin.

The examination of the reactions of the anthocyanidins in a range of buffered solutions has shown that this method is by far the most reliable for purposes of comparison and characterisation; it is of even greater value in this connection than a study of the absorption spectrum.

On the one hand various properties—*pseudo*-base formation and colour-base precipitation, ease of oxidation and so forth, are incidentally revealed and on the other, different anthocyanidins, as for instance, peonidin and malvidin, may exhibit identical absorption spectra [Schou, 1927].

A few examples have been selected to illustrate the method and it is hoped that the data will be found useful for the identification of anthocyanidins derived from natural sources.

Fear and Nierenstein state that cyanidin chloride from cyanin behaves differently from synthetic 3 : 5 : 7 : 3′ : 4′-pentahydroxyflavylium chloride and it is evident that by some misfortune they have not succeeded in obtaining a homogeneous specimen of the latter substance.

We repeat an emphatic affirmation that pure synthetic 3 : 5 : 7 : 3′ : 4′-pentahydroxyflavylium chloride, best prepared by hydrolysis of its benzoyl derivative, exhibits no divergences from cyanidin chloride and this is true, to the minutest detail, in regard to the tests described below.

Indeed the constitution of cyanidin chloride in its main structural outlines (carbon and oxygen skeleton) is established as firmly as that of any other compound and no attempt [Malkin and Nierenstein, 1928] to reconcile the results of Nierenstein [1921] in the field of catechin chemistry with the conflicting results of Freudenberg and Purrman [1924], by suggesting a modified cyanidin formula, can succeed. The explanation of the discrepancies must be sought elsewhere.

Experimental.

The buffered solutions were prepared from the tubes of "Universal Buffer Mixture," supplied by the British Drug Houses Limited. This mixture is stated to be compounded in accordance with a formula of Prideaux and Ward [1924] but the exact composition has not been disclosed.

It may happen that this excellent commercial product may be unobtainable in some quarters or at some future time and as it is in any case necessary to guarantee the reproducibility of our results we think it well to mention that a mixture of 0·04 g. mol. each of phenylacetic acid, boric acid and potassium dihydrogen phosphate appeared to be the equivalent of the contents of one of the tubes supplied. Phenylacetic acid, boric acid and potassium dihydrogen phosphate (0·02 g. mol. each), together with n cc. of 0·2 N sodium hydroxide, were dissolved in water and made up to 1000 cc. The solutions used and the approximate p_H of each, as found by means of indicators, were the following: (1) $n = 0$; p_H, 3·2; (2) $n = 25$; p_H, 3·8; (3) $n = 50$; p_H, 4·4; (4) $n = 75$; p_H, 5·0; (5) $n = 100$; p_H, 5·6; (6) $n = 125$; p_H, 6·2; (7) $n = 150$; p_H, 6·8; (8) $n = 175$; p_H, 7·4; (9) $n = 200$; p_H, 8·0; (10) $n = 225$; p_H, 8·6; (11) $n = 250$; p_H, 9·2; (12) $n = 275$; p_H, 9·8; (13) $n = 300$; p_H, 10·4; (14) $n = 325$; p_H, 11·0; (15) $n = 350$; (16) $n = 400$; (17) $n = 450$.

The anthocyanidin chloride (25·00 mg.) was dissolved in 98 % alcohol (100 cc.) and 1 cc. of the solution added from a burette to 10 cc. of the buffered solutions or of other solutions mentioned below.

Apigeninidin chloride. The alcoholic solution was orange; 1 % hydrochloric acid, bright canary yellow unchanged after 24 hours; 20 % hydrochloric acid, bright canary yellow and after 24 hours almost the whole of the salt had separated in clusters of microscopic prisms; (1) more orange yellow than the solution in 1 % hydrochloric acid, unchanged after 24 hours; (2) more intense yellowish orange, slight fading after 24 hours; (3) reddish orange fading slightly during 24 hours; (4) orange red, after 24 hours, long, slender crystals of colour base deposited; (5) darker orange red, after 24 hours colour base crystallised in slender needles; (6) brownish red, after 24 hours slight fading; (7) brownish red, a little bluer, same as (6) on keeping; (8) still a little

bluer brownish red, same as (6) on keeping; (9) bluish red, same as (6) on keeping; (10) cherry red, bluer than (9) and became like (6) on keeping; (11), (12), (13), (14), (15) same as (10); (16) same as (10) but became reddish orange after 24 hours; (17) same as (10) but became greenish yellow after 24 hours.

Pelargonidin chloride. 1 % and 20 % hydrochloric acid, orange red with bluish tinge in thin layers; (1) identical with hydrochloric acid solutions, colour faded but more slowly than in (2), (3) and (4), in 2 hours very faintly coloured, in 3½ hours colourless and remained so; (2) a little browner orange red, almost all colour disappeared in 17 minutes and colourless in 30 minutes; (3) bluish red fading quickly to pink and became colourless a little more rapidly than (2); (4) cherry red fading rapidly, still more so than (3); (5) bluish red, after 15 minutes rose coloured, after 3½ hours colourless (it should be noted that observations at all intermediate times could not be made and, for example, this solution may have been colourless after 2 hours); (6) violet red, after 3½ hours pale pink and after 24 hours still very weak red colour; (7) slightly bluer violet red, subsequent changes like (6); (8) like (7), after 3½ hours faded brownish pink, more intense than (7); (9) deeper violet, after 25 minutes reddish violet, after 24 hours weak reddish brown whereas (8) at this stage had a much weaker red colour; (10) violet, blue in thin layers, in 5 minutes pale violet, in 25 minutes reddish violet, after 24 hours weak reddish violet; (11) a little bluer than (10), after 25 minutes reddish violet, after 24 hours weak reddish violet; (12) a little bluer than (11) and dichroism less pronounced, after 10 minutes violet, after 25 minutes red violet, after 24 hours weak reddish violet; (13) again bluer, after 10 minutes violet, after 25 minutes red violet and after 24 hours very weak reddish brown; (14) similar, thin layers are greener blue, after 10 minutes violet, after 25 minutes violet, after 3½ hours weak reddish violet, after 24 hours very faint orange; (15), (16), (17) similar to (14) on mixing and after 24 hours, but became pale grey blue in 3 hours.

Cyanidin chlorides. 1 % and 20 % hydrochloric acid, eosin red, much bluer than pelargonidin; (1) red, slightly bluer than 1 % hydrochloric acid; the colour faded very slowly, after 18½ hours colourless; (2) bluish red, after 2½ minutes colour distinctly less intense, after 10 minutes considerable fading, after 45 minutes colourless; (3) reddish violet, after 10 minutes very weak, after 25 minutes colourless whereas at this stage both (2) and (4) retained weak colour; (4) bluer reddish violet, after 10 minutes colour much faded, after 45 minutes still weak violet, after 18½ hours colourless; (5) reddish violet, still bluer than (4), 15 seconds after mixing the colour-base separated giving opalescent solution; after 18½ hours precipitate of violet colour-base and colourless supernatant liquid; (6) bluer reddish violet of increased tinctorial intensity, no precipitate, after 18½ hours almost colourless—very faint pink; (7) violet tinged with red, after 18½ hours very faint pink; (8) very like (7); (9) again similar but a little bluer and already blue in very thin layers, after

8 minutes became weak grey violet, after 18½ hours almost colourless; (10) much bluer violet and pure blue in thin layers, after 18½ hours extremely weak grey; (11) similar to (10) but less dichroic, not so red violet in thicker layers, after 18½ hours same as (10); (12) same as (11); (13) same as (11), after 18½ hours pale orange yellow; (14) less dichroic than (11), after 18½ hours more intensely yellow than (13); (15) still less dichroic and still deeper yellow on keeping 18½ hours; (16) greenish blue, after 30 minutes colour largely disappeared, after 18½ hours deeper yellow than (13); (17) bluish green, after 8 minutes almost colourless, after 30 minutes orange yellow, after 18½ hours deeper yellow than (13).

5-O-*Benzoylcyanidin chloride*. The alcoholic solution was the colour of an aqueous permanganate solution. 1 % hydrochloric acid, bluish red of no great tinctorial intensity, after 30 minutes faint pink, after 1½ hours colourless; 20 % hydrochloric acid, the salt was precipitated leaving a colourless solution; (1) bluish red rapidly fading and colourless after 1 minute; (2) bluish red, after 12 minutes colourless; (3) similar but still slower fading, after 12 minutes faint pink, after 30 minutes colourless; (4) similar, a little slower fading; (5) red violet, after 12 minutes weak violet pink, after 30 minutes pale reddish violet, after 24 hours colourless; (6) more intense violet fading somewhat rapidly, after 12 minutes very pale violet, after 24 hours still a violet tinge; (7) very similar to (6) but faded a little less throughout; (8) similar to (7) and fading a little less throughout; (9) blue violet, did not fade very rapidly but became more violet; (10) blue with violet tinge becoming more violet, after 12 minutes violet blue, after 30 minutes violet, after 24 hours reddish yellow; (11) blue with slight violet tinge, subsequent changes like (10); (12) pure blue in thin layers, after 6 minutes violet blue, after 25 minutes blue violet, after 24 hours yellow; (13) same as (12) but became violet a little more slowly; (14) pure blue, after 20 minutes green, after 24 hours yellow; (15) pure blue, after 5 minutes green, after 20 minutes greenish yellow, after 24 hours yellow; (16) greenish blue changing through green to yellow more quickly than (15); (17) similar to (16).

Peonidin chloride. 1 % and 20 % hydrochloric acid, eosin red, same shade as cyanidin, unchanged after 18½ hours; (1) eosin red a little bluer than hydrochloric acid solutions, after 15 minutes faded and after 24 hours colourless; (2) cherry red, fading is most rapid at this p_H, after 30 minutes colourless; (3) bluer red, after 2 minutes browner red fading rapidly, after 15 minutes the colour was weak, after 11½ hours colourless; (4) bluish red which quickly faded and became browner, after 18½ hours some colour-base had separated; (5) very much like (4); (6) reddish violet which did not fade so quickly as (5), (4), (3) and (2), after 18½ hours some colour-base had separated; (7) violet with reddish tinge, bluer than (6), fading comparatively slowly, after 18½ hours faint pink and no precipitate; (8) violet, after 18½ hours faint reddish violet; (9) bluish violet, blue in thin layers, after 18½ hours weak violet; (10) blue, violet in thick layers, after 18½ hours bluish grey; (11) a little greener blue

becoming still greener in 1 minute, after 18½ hours bluish grey; (12) like (11), after 18½ hours greenish grey; (13) greenish blue, after 18½ hours weak yellow; (14), (15), (16), (17) almost same as (13) but curiously the fading in (16) and (17) was less rapid than in (14) and (15); this suggests that both oxidation and *pseudo*-base formation are involved.

Malvidin chloride. 1 % and 20 % hydrochloric acid bluish red, unchanged after 24 hours; (1) a little bluer red, weak pink after 20 minutes, colourless after 50 minutes; (2) similar to (1) but fading more rapidly, almost colourless after 20 minutes; (3) bluish red at once changing to pale brownish cherry red, after 5 minutes pale rose, almost colourless after 20 minutes; (4) similar to (3); (5) similar to (3) and (4) at first but fading is most rapid and in less than 15 minutes there was a brown violet red precipitate in a colourless solution, after 3 hours there was still a flocculent precipitate but after 5 hours all the solid had passed into solution; (6) violet fading rapidly with a slight precipitation; almost colourless after 3 hours; (7) pure violet solution blue in thin layers and no precipitate, after a few minutes faded violet blue, after 3 hours pale blue, after 24 hours almost colourless; (8) blue, violet in thick layers and rapidly fading, after 10 minutes the series (8), (9), (10), (11), (12) had all faded similarly and there was little divergence in the colours which ranged from weak violet blue to weak greenish blue, after 2½ hours the series (8) to (12) were very pale blue to greenish blue, after 24 hours (10), (11) and (12) were pale orange; (9) pale blue fading rapidly; (10) greenish blue fading rapidly; (11) similar to (10); (12) similar to (10); (13) greenish blue becoming greener as it faded, bluish green after 30 minutes, the change to green occurred more and more rapidly in the ensuing series (13) to (17), on keeping changes like (10); (14) similar, yellowish green after 1½ hours; (15) similar, greenish yellow after 1½ hours; (16) similar, brownish yellow after 1½ hours; (17) similar.

Cyanin chloride. In the case of anthocyanins, 25·00 mg. were dissolved in 100 cc. of methyl alcoholic hydrochloric acid (1 cc. aqueous hydrochloric acid, sp. gr. 1·16, made up to 1000 cc. with methyl alcohol); the acid thus introduced causes a reduction of 0·1 in the p_H of all the solutions. The specimen of cyanin chloride employed was derived from deep-red dahlia flowers and was kindly sent to us by Professor R. Willstätter, to whom we are greatly indebted.

Solutions in 1 % and 20 % hydrochloric acid were salmon red; (1) pink, much less intense owing to *pseudo*-base formation; (2) a little bluer than (1) and rapidly faded; (3) bluish red fading rapidly; (4) permanganate-coloured fading rapidly; (5) similar, fading less quickly; (6) similar but colour more intense and fading quite slowly; (7), (8), (9) similar to (6); (10) reddish violet; (11) violet; (12) violet; (13) bluer violet; (14) violet blue fading quickly; (15) blue fading quickly; (16) greener blue fading rapidly; (17) similar.

After 15 to 20 minutes (1) was pink and the colour diminished in the series to (5) which was colourless; (6) had faded but was intense in comparison with (1); (7), (8), (9), (10), (11) and (12) had faded somewhat and formed

a series ranging from violet red to violet; (13) was weak violet; (14) weak violet blue and (15), (16) and (17) were almost colourless (grey).

After 5 hours (1) was feebly pink and the colour diminished to (5) which was colourless, then gradually increasing reddish violet to (10) which was however very pale coloured; (11) was similar but not so clear toned; (12) to (17) inclusive were pale yellow.

After 20 hours the solutions in 1 % and 20 % hydrochloric acid were unchanged; (1) was pink and the colour in the range diminished to (5) which was colourless, then increased to (9) which was weak pink; (10) was weak brownish pink and (11) and the remainder were pale yellow.

Malvin chloride. We are greatly indebted to Professor P. Karrer for the provision of a specimen of this diglucosidic anthocyanin. The solutions in 1 % and 20 % hydrochloric acid were bluish red; (1) bluish red becoming pale rose in a few seconds; (2) quickly decolorised; (3) same as (2); (4) bluish red fading in the course of 2 minutes; (5) reddish violet fading slowly; (6) violet fading slowly; (7) bluish violet; (8) violet blue; (9) blue; (10), (11) and (12) similar to (9); (13) blue fading to greenish blue; (14), (15), (16), (17) blue fading with increasing rapidity to pale green.

After 20 minutes (1) was pink, (2), (3), (4) and (5) were colourless, (6) was weak reddish violet, (7) was a little deeper violet; (8) was bluish violet; (9), (10), (11) and (12) were blue; (13) was greenish blue; (14) was bluish green; (15) was yellowish green; (16) was greenish yellow, and (17) was yellow.

After 5 hours (1) to (8) inclusive were almost colourless ((1) was tinged pink, (7) reddish violet, and (8) pale blue); (9) was weak violet blue; (10) was weak pure blue; (11) was pale greenish blue; (12) was pale green, and (13) to (17) inclusive were yellow.

After 20 hours (1) was faint pink; (2) to (6) inclusive were colourless; (7) was very faint pink; (8) was very faint blue; (9) was pale blue; (10) was blue; (11) was bluish green, and (12) to (17) were yellow. The solutions in 1 % and 20 % hydrochloric acid were unchanged.

Other anthocyanins have been similarly characterised and the results will be subsequently recorded in connection with synthetical experiments.

It should be stated that all the tests were carried out in the summer and that the room temperature was 20–25°; no precautions were taken to exclude air.

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VII. THE RELATION BETWEEN CYSTINE YIELD AND TOTAL SULPHUR IN WOOL.

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(*Received December 24th, 1928.*)

In an investigation now in progress upon the nature of the sulphur linkage in wool, it became important to know whether the whole of the sulphur could be accounted for as cystine, or whether there existed other, hitherto undiscovered, amino-acids present in the protein molecule and containing the element sulphur in their constitution. Suggestions of such a nature have been made from time to time.

The kind of evidence usually brought forward as indicative of the presence of sulphur-containing substances other than cystine is divisible, broadly, into arguments of two kinds. First, differences have been noted and emphasised in the manner of reaction, towards alkaline solutions, of cystine and proteins. The latter lose all or a part of their sulphur with comparative ease, the sulphide ion being formed; cystine solutions under similar conditions suffer far less change. Moreover the fraction of the total sulphur which is eliminated as sulphide is not the same in all proteins but varies between wide limits [see Osborne, 1900; Fleitman, 1847, 1848; Krüger, 1888; Mörner, 1901].

The second class of arguments was based on the fact that in no case does the yield of cystine obtained from a protein after hydrolysis approach the value to be expected, were its sulphur present solely in this form. This aspect of the matter has been dealt with by Harris [1923] and by others. Cystine is not an easy amino-acid to isolate in quantitative yield, even from pure solutions, as Harris has shown, and its solubility, and therefore the difficulty of its separation, is enormously increased by the presence of other amino-acids [cf. Hopkins, 1921].

The methods available for its isolation have been reviewed by Barritt [1927], whose work represents the most recent attempt to obtain quantitative isolation from wool. Even under the most suitable conditions, in no case was he able to obtain more cystine than corresponded to 66 % of the total sulphur.

Since the work of Abel and his associates upon the sulphur of insulin, the question of the ratio of "labile" to "firmly bound" sulphur in proteins has taken on a new interest. In this connection Brand and Sandberg [1926] have

made the important observation that lability of the sulphur atom towards alkali differs enormously in the different compounds of cystine. Results of a similar kind have been obtained by Bergmann and Stather [1926]. On the other hand, du Vigneaud, Jensen and Wintersteiner [1928], working with crystallised insulin, found that after acid hydrolysis only about one half of the total sulphur could be accounted for as cystine, using the Sullivan [1926] colorimetric method for the determination of the latter, whilst Folin, and Looney's [1922] colorimetric reagent gave a value consistently higher and approximating closely to that of the total sulphur. The Sullivan method is—so far as is known—specific for cystine, whilst the Folin and Looney reagent develops a colour with all substances in which the S—S linkage is present. Hence it seemed probable to du Vigneaud and his collaborators that there was present in insulin a substance which was not cystine but which contained sulphur in disulphide linkage.

Accordingly, for the purposes of the present investigation, it was decided to carry out cystine determinations upon acid hydrolysates of wool samples of different origin, using the two colorimetric methods mentioned above, and to compare the results so obtained with the cystine content reckoned by assuming all the sulphur of the wool to be present as cystine.

In every case examined a very close agreement has been found between these two values, all the sulphur of the wool being accountable as cystine measured by the Sullivan method (the most specific, short of direct isolation). The Folin and Looney values were found consistently to be slightly higher than the Sullivan values—and higher than the total sulphur—but this reagent is known to give a blue colour with tyrosine, hydroxytryptophan and other amino-acids.

The conclusion is drawn, therefore, that the whole of the sulphur of wool is liberated as cystine when the protein is hydrolysed by acid. The method is clearly applicable to all other proteins.

Experimental.

Raw wool samples were used in every case. These were degreased by washing twice in warm (50°) benzene, air-dried, and then washed thoroughly in cold 0·1 % saponin solution. Washing was effected by rinsing in many successive changes of distilled water, using a relatively large volume for each rinse. The wool was again air-dried by leaving it spread out very thinly in a warm dry atmosphere for several days. Samples were then taken for sulphur and moisture (regain) determination and at the same time for hydrolysis. Moisture content was determined by recording the loss in weight when a current of dry air was led over the sample contained in a specially designed drying bottle and held at a temperature of 105°. Total sulphur was determined by the Benedict-Denis method as used by Trotman and Bell [1926], or by the Carius method.

For hydrolysis 6 g. of air-dried wool were weighed out and added to 100 cc. of boiling 20 % hydrochloric acid. Boiling was maintained, using a sand plate and reflux condenser and samples of 5 cc. were withdrawn at intervals of $\frac{1}{4}$, $\frac{1}{2}$ hour, and thence at every hour from the time of commencement. Each sample was pipetted into a 50 cc. volumetric flask, diluted largely with water, and then rendered nearly neutral by the cautious addition of 5 cc. of 20 % sodium hydroxide, after which the total volume was made up to 50 cc. and the sample filtered. For cystine determination according to the Sullivan method 2 cc. of the sample were used, and a suitable amount of a standard 0·05 % cystine solution in 0·1 N hydrochloric acid, water being added to the standard if necessary to bring the volume to 2 cc. To each, 1 cc. of a 5 % sodium cyanide solution was added and after exactly 10 minutes 1 cc. of a 0·5 % solution of 1:2-naphthoquinone-4-sodium sulphonate and 5 cc. of 10 % sodium sulphite in 0·5 N sodium hydroxide. After 15 minutes each flask received 1 cc. of 2 % sodium hydrosulphite in 0·5 N sodium hydroxide. Colours were compared in a Bausch and Lomb colorimeter. It was found by experience that results were not dependable unless standard and unknown solutions contained very nearly the same quantity of cystine, and therefore a separate standard was made up for determination of each sample, allowing a difference in reading of not more than 15 %. The sulphite, hydrosulphite and quinone solutions were always freshly prepared, never being more than four hours old.

Using these precautions the method gave very satisfactory results. Since long boiling of cystine with strong acids converts it into an isomeric form differing from true cystine in crystalline form and in being optically inactive [Hoffman and Gortner, 1922], it was thought necessary to prepare some of this isomeric cystine and ascertain whether it was estimable by the Sullivan method. Accordingly 10 g. of cystine were boiled under reflux with 150 cc. of 20 % hydrochloric acid for 144 hours. The solution was filtered, decolorised with charcoal and the cystine precipitated by bringing to p_H 3·7 with sodium acetate. After recrystallisation it was analysed and compared with ordinary cystine by the Sullivan method. It gave 99·5 % of the colour of true cystine, an agreement which is well within the sensitivity of the method.

The Folin and Looney method was carried out employing 2 cc. of the test solutions and 2 cc. of the cystine standard. To each, 3 cc. of water were added, followed by 5 cc. of N sodium hydroxide and 10 cc. of fresh 20 % aqueous sodium sulphite. After 5 minutes, 3 cc. of the Folin and Looney reagent were added, and after waiting another 10 minutes the solutions were made up to a total volume of 25 cc. and compared.

The cystine used as standard was analytically pure (S = 26·79 %).

Total sulphur was determined upon 5 cc. of the hydrolysate by evaporating the hydrochloric acid in a silica dish on the water-bath adding 5 cc. of Benedict-Denis reagent and proceeding in the usual manner.

A typical result is shown in the curve, Fig. 1 (Crossbred 50's wool). The

liberation of free cystine proceeds rapidly and is complete within 5 to 7 hours. There is some indication of a fall after the maximum cystine value has been reached. This is in agreement with the findings of Merrill [1921], who also obtained the greatest yield of cystine from wool after 5 to 7 hours' hydrolysis. This destruction of cystine probably accounts for the fact that the maximum value is generally about 0·5 % short of the total sulphur figure reckoned as cystine. It hardly seems likely that any other amino-acid should be responsible for this very slight amount of extra sulphur. The values obtained by the Folin and Looney method correspond closely with the maximum Sullivan value with the exception of the first three or four points (1 to 2 hours). The fall from a value some 10 % higher than the final steady line is a constant feature observed in every experiment and can only be interpreted as indicating the formation of substances (possibly of phenolic nature) to which the reagent is

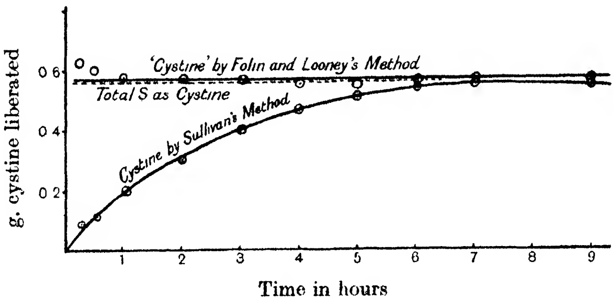

Fig. 1. Hydrolysis of wool (Crossbred 50's) by 20 % HCl.

sensitive and which are comparatively rapidly destroyed. Abderhalden and Fuchs [1913] have shown that tryptophan and hydroxytryptophan give a blue colour with the phosphomolybdate-tungstate reagent; they may equally well react in the present circumstance. It is well known that these substances are not stable under the conditions of an acid hydrolysis. The Folin and Looney values, it will be noticed, do not show a gradual increase in cystine as hydrolysis proceeds, but reach their maximum directly the wool is dissolved (within 10 minutes). As the reagent reacts with the disulphide linkage it would naturally react with cystine peptides and hence gives the form of curve to be expected.

Most of the preliminary work was done upon a Crossbred 50's wool, S = 3·46 %, but among other samples examined were a typical low sulphur wool—Devon lamb's wool, S = 3·34 %, and a high sulphur wool—Welsh mountain[1], S = 4·08 %, and also a fine and a coarse Turkey mohair. No difference in behaviour was found between these and other wools. The following table shows the results obtained.

[1] For these samples and the determination of their total sulphur contents, I am indebted to Mr Barritt, of this Association.

Table I.

Wool	Total S % dry wt.	Cystine in hydrolysate: By Sullivan's method (max. value) g.	By Folin and Looney's method g.	Total S of hydrolysate (as cystine) g.	Ratio*
Crossbred 50's I	3·46	0·3780	—	0·3602	+4·94
,, ,, II	3·47	0·5700	0·5950	0·5815	-1·99
,, ,, III	3·54	0·5575	0·5673	0·5589	-0·25
Devon lamb	3·34	0·5755	0·5825	0·6001	-4·10
Welsh mountain	4·08	0·6300	0·6400	0·6277	+0·37
Turkey mohair (coarse)	3·02	0·5978	0·6080	0·5948	+0·50
Turkey mohair (fine)	3·18	0·5405	0·5564	0·5393	+0·22
Cape merino	3·67	0·5875	0·5963	0·5887	-0·02

$$* \frac{\text{Total S (hydrolysate)} - \text{cystine (Sullivan)} \times 100}{\text{Total S}}.$$

Precipitation of cystine by phosphotungstic acid.

Since du Vigneaud, Jensen and Wintersteiner [1928], found that their non-cystine, sulphur-containing substance could be approximately separated from cystine by precipitation of the latter with phosphotungstic acid, it was decided in the present case to adopt a similar procedure in order to check the results obtained.

40 cc. of an acid hydrolysate—containing in all 232 mg. of cystine—were diluted to 240 cc. and 15 g. of phosphotungstic acid added. The flask was stoppered and immersed in ice for 24 hours. The phosphotungstates were then filtered off, washed and dissolved in sodium hydroxide. Phosphotungstic acid was removed from this solution and from the filtrate by barium chloride in the usual way and excess of barium by sulphuric acid. Determinations were then carried out by the Sullivan and Folin and Looney methods and yielded the following results.

	Phosphotungstate precipitate	Filtrate
Total cystine by Sullivan method.	117·5 mg.... ...	55·0 mg.
Total cystine by Folin and Looney method.	120·6 mg.	65·0 mg.
Total tyrosine by Folin and Ciocalteu method [1927]		100·8 mg.
Equivalent colour as cystine[1]		9·4 mg.

Wool contains about 3 % of tyrosine. The quantity present in this experiment gives an equivalent cystine value with the Folin and Looney reagent of 9·4 mg. in the filtrate. Tyrosine alone would therefore account almost entirely for the discrepancy observed between the figures in the last column. A second experiment gave similar results.

Determination of sulphur in wool from cystine yield.

From the quantity of cystine formed when a sample of wool is boiled with hydrochloric acid, the amount of sulphur originally present in the wool may

[1] 10 mg. tyrosine were found to give the same colour with the Folin and Looney reagent as 0·93 mg. of cystine.

be determined. The following example is taken from an actual experiment using a Crossbred 50's wool.

A known weight of wool was boiled for 7 hours in a Kjeldahl flask with 20 % hydrochloric acid. When cool, the hydrolysate was neutralised by sodium hydroxide, the solution filtered and, together with the washings, made up to 100 cc. Cystine was determined in 2 cc. of this liquid using the Sullivan method.

The moisture content of the wool was ascertained by weighing out a sample at the same time that the sample for hydrolysis was taken and proceeding as described earlier in this paper.

The following figures were obtained.

Weight of wool hydrolysed	0·5183 g.
Moisture content	13·27 % of wet wt.
Cystine in acid hydrolysate	57·75 mg.

whence

Sulphur content of dry wool	3·43 %.

The sulphur content by the Carius method was found to be 3·46 %.

Summary.

It may be concluded from these experiments that natural wools differing markedly in their sulphur content (3·34 % to 4·08 %—nearly the extremes of variation encountered) and also fine and coarse samples of Turkey mohair are all capable of yielding their entire sulphur as cystine when hydrolysed by acids. There is no evidence of any other sulphur compound entering into their constitution.

The author's acknowledgments are made to Mr A. T. King, Chief Chemist to the Association, for help and advice rendered during the course of the work.

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VIII. A SPECIFIC COLOUR REACTION FOR ERGOSTEROL.

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THE unique function of ergosterol as the parent substance of vitamin D made it desirable to find a colour reaction for it, by means of which it could be detected in the presence of other sterols. In searching for such a reaction, the property of formaldehyde of shifting the colour from the red into the blue part of the spectrum in the usual colour reactions of sterols [Whitby, 1923; Rosenheim, 1927] suggested an investigation of the behaviour towards sterols of the aldehyde corresponding to trichloroacetic acid. This acid is known to give rise to colour reactions with cholesterol [Tschugajeff, 1900].

Although anhydrous chloral proved to be non-reactive, it was found that both chloral hydrate and trichloroacetic acid give a characteristic blue colour reaction with ergosterol, whilst all the other naturally occurring sterols investigated, when purified from ergosterol, remain colourless under the same conditions. The specimens of ergosterol used in these experiments had been fractionally recrystallised at 36° and possessed the highest optical activity, $[\alpha]_D - 132°$, so far recorded [Tanret, 1908; see also Bills and Honeywell, 1928]. The reactions to be described are therefore unlikely to be due to impurities or degradation products of ergosterol.

In contradistinction to naturally occurring sterols it was found, when studying the reaction of sterol derivatives, that the production of an immediate red colour with either of these reagents is specific for the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage of the sterol ring system. These observations suggest an explanation of the mechanism of sterol colour reactions, which is applicable to all of them and will be discussed later.

I. *Chloral hydrate reaction.*

When a few crystals, 1 mg. or less, of ergosterol are added to about 0·5 g. chloral hydrate, liquefied by warming in a water-bath, they dissolve and immediately give rise to a carmine red solution, showing a broad absorption band at 500 $\mu\mu$. The red colour changes within a minute into a green and finally into a deep blue, which persists for a considerable time. The esters of ergosterol react in the same way. The colour is discharged rapidly

by water or alcohol, more slowly by chloroform, benzene, toluene or other anhydrous solvents not possessing a hydroxyl group.

Although the blue colour is discharged by dilution with water, a saturated aqueous solution of chloral hydrate (80 %) reacts in the typical manner when a drop of concentrated HCl is added. It would therefore appear that traces of an acid are essential for the reaction.

Freshly distilled anhydrous chloral dissolves ergosterol. The colourless solution undergoes the above-described changes on the addition of one drop of water. On keeping under laboratory conditions, chloral, or its chloroform solution, attracts moisture and gradually becomes "activated," so as to give the reaction with ergosterol without the addition of water.

Colourless solutions are given under these conditions by all the other naturally occurring sterols, their esters and their reduction products. The following carefully purified sterols were examined: cholesterol, sitosterol, γ-sitosterol (1 double bond); stigmasterol (2 double bonds); zymosterol, fungisterol (3 double bonds); *iso*cholesterol (saturated?), amyrol, coprosterol and dihydrositosterol (saturated). In the preparation of zymosterol from yeast and fungisterol from ergot [Rosenheim and Webster, 1928], it was found that the crude specimens gave the reaction strongly. The intensity of the reaction decreased, however, proportionately to their progressive purification and the removal of ergosterol. The agreement of the spectroscopic and biological examination with the colorimetric results justifies the conclusion that the slight positive reaction of the purest specimens of zymosterol and fungisterol obtained so far is due to admixture of ergosterol.

II. *Trichloroacetic acid reaction.*

An aqueous solution, prepared by dissolving nine parts of the pure crystallised acid in one part water, was found to be the most suitable reagent. When this is added to ergosterol dissolved in a few drops of chloroform, an immediate red solution (band at $500\mu\mu$) is produced, which changes gradually into a clear blue (bands at 570–580 and at $650–680\mu\mu$), without showing the intermediate green phase of the chloral hydrate reaction. In distinction from the latter, the reaction takes place at ordinary temperature and has the further advantage of yielding a final blue solution, which may be diluted for colorimetric purposes with the reagent itself or with chloroform. Excessive dilution with chloroform or other solvents tends to change the colour into a bluish-green of the same shade as that given by ergosterol with a saturated chloroform solution of trichloroacetic acid. The latter solution undergoes decomposition on keeping, liberating phosgene and hydrochloric acid, and is not so suitable a reagent as the aqueous solution.

The sensitiveness of the reaction was determined by adding three drops of the reagent to 0·1 cc. of a chloroform solution containing known amounts of ergosterol. It was found that 0·01 mg. ergosterol still gave a marked reaction

within 5 minutes, and that the colour is just recognisable with 0·005 mg. when compared with the colourless control. The sensitiveness is therefore of approximately the same order as that of the usual sterol reactions.

Employing mixtures of cholesterol and ergosterol, prepared by mixing their solutions in chloroform, a strong reaction is obtained in the presence of 0·5 % ergosterol in 0·1 g. cholesterol, and even smaller quantities down to 0·1 % can be detected by comparison with the colourless cholesterol control. The amount of ergosterol in ordinary cholesterol, as estimated by the spectroscopic test, may vary from 0–0·1 % according to the method of purification used and depending on the source of the sterol. Cholesterol prepared from brain or cod-liver oil, having undergone charcoal treatment, may occasionally be free from ergosterol [Rosenheim and Webster, 1927], whilst as much as 0·12 % may be present in preparations from spinal cord [Bills, Honeywell and MacNair, 1928]. Examination of a large number of "pure" specimens, M.P. 147–148°, prepared from brain, gallstones, liver, spleen, skin, blood, ovaries (pig), eggs (frog) and cod-liver oil gave negative tests in some cases (brain, gallstones) and positive reactions in most. The intensity of the reaction in descending order was approximately: eggs (frog), ovaries, liver, skin (pig), brain, spleen, blood. Specimens of sitosterol from wheat and maize also gave positive reactions before purification by the bromine method. Whilst the colour reaction for ergosterol in sterols probably does not equal that of the biological or spectroscopic test in sensitiveness, it affords chemical evidence for the assumption that the impurity in ordinary cholesterol, which gives rise to vitamin D on irradiation, is identical with ergosterol.

The other naturally occurring sterols examined (see above) do not react with the freshly prepared reagent and remain colourless for more than 30 hours when kept at room temperature in the dark. On warming, however, cholesterol [Tschugajeff, 1900; Hirschsohn, 1902] and the other unsaturated sterols rapidly give a red solution, showing an absorption band at 500 $\mu\mu$.

A specific reaction for the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage in sterols.

A study of the behaviour of the two reagents towards cholesterol derivatives, in which known chemical changes had been produced, showed that esterification of the hydroxyl group, its replacement by chlorine, or its complete removal, has no effect, for cholesterol acetate or chloride and cholestene gave colourless solutions. Reduction or bromination, etc., of the double bond are equally without influence, since dihydrocholesterol, cholestane, cholesterol dibromide and cholesterol oxide are also non-reactive. The doubly unsaturated ketone, "oxycholesterilene" and dicholesteryl ether, as well as digitaligenin, also gave negative results.

On applying the chloral hydrate and the trichloroacetic acid reaction to *allo*cholesterol and *allo*sitosterol, however, an immediate yellow → orange colour was produced, which rapidly deepened into a permanent carmine red. In both cases the red solutions showed an absorption band at 500 $\mu\mu$. In

the $AsCl_3$ and $SbCl_3$ reactions, the behaviour of the *allo*-compounds is identical with that which they show with the above reagents.

The conversion of cholesterol into the isomeride *allo*cholesterol was effected by means of hydrochloric acid by Windaus [1927], who also showed conclusively that a shifting of the double bond from $C_{6,7}$ to $C_{1,2}$ (or $C_{1,13}$) takes place under these conditions (see formulae I and II, p. 52).

The above results indicate that an immediate red colour reaction is directly dependent on the presence of the $\Delta^{1,2}$ linkage[1], and it seemed therefore of interest to test the behaviour of other sterol derivatives, in which the existence of the same linkage had been postulated. Heilbron and Sexton [1928] arrived at the conclusion that "one of the ethenoid linkages in cholesterilene must occupy the same position as in ψ-cholestene," which latter substance possesses only one double linkage, presumably in the same position as in *allo*cholesterol. Specimens of cholesterilene, ψ-cholestene and ψ-cholestane, prepared by these authors, were kindly put at my disposal by Prof. Heilbron, and it was found that both cholesterilene and ψ-cholestene gave an intense red reaction, the latter somewhat more slowly than the former. On the other hand, ψ-cholestane remained colourless, as was to be expected in the case of this saturated hydrocarbon.

The positive results confirm Heilbron and Sexton's conclusions, and may be taken as supplementary evidence for the specificity of the reaction. They lead further to the suggestion that the primary red phase of the ergosterol reaction (see above) is due to the presence of the $\Delta^{1,2}$ linkage. This conclusion is in agreement with that reached by Heilbron, Morton and Sexton [1928] from their studies of the ultra-violet absorption spectrum of cholesterilene, which led them to infer "that of the three double bonds in ergosterol, two occupy the same position as in cholesterilene." The final blue stage of the ergosterol reaction may therefore justifiably be ascribed to the influence of the third double linkage, the position of which is at present unknown.

It is of interest to note further that an immediate red reaction is also given by β-cholesterol, an isomeride of cholesterol obtained by Diels and Abderhalden [1908], by heating cholesterol to 310°. The position of the double bond in this isomeride remains unknown. It seems permissible to infer from the positive result of the above colour reaction that β-cholesterol also contains the $\Delta^{1,2}$ linkage, its isomerism with *allo*cholesterol being due to the attachment of the hydrogen atom to the carbon atom C_2 (or C_{13}) in either the *cis*- or *trans*-position.

The bathychromic effect of formaldehyde. In two colour reactions of cholesterol, namely with concentrated H_2SO_4 and with $AsCl_3$, formaldehyde has a bathychromic effect, *i.e.* it deepens the colour from red to violet [Whitby, 1923; Rosenheim, 1927]. The same effect is observed in the trichloroacetic acid reaction with *allo*cholesterol and *allo*sitosterol: if a few drops of formalin

[1] For convenience of discussion, the symbol $\Delta^{1,2}$ only is used in the following, implying also the possibility of the $\Delta^{1,13}$ linkage.

are added when the red phase has been reached, the colour changes gradually into a purple and violet. The reaction is conveniently carried out with a saturated solution of the acid in formalin as a reagent, which produces rapidly a violet reaction with the *allo*-compounds, showing a band at 590–610 $\mu\mu$. Cholesterol behaves in the same way with trichloroacetic acid and formalin, but heating in this case is necessary [Golodetz, 1908]. In the reaction with ergosterol, on the other hand, formaldehyde exerts a hypsochromic effect, and prevents the formation of the blue phase. When added to the reaction mixture at the blue stage, it gradually weakens and discharges the colour.

It has been shown previously [Rosenheim, 1927] that, on heating cholesterol in chloroform solution with benzoyl peroxide, the white product obtained gives a blue colour with the reagents which usually give rise to a red colour. When similarly treated, *allo*cholesterol also gives a blue colour with trichloroacetic acid. The gentian-blue colour of this reaction is, however, easily distinguished by the naked eye from the colour of the ergosterol reaction (starch-iodine-blue), and spectroscopic examination shows in the former solution the characteristic band of "oxycholesterol" only. The blue reaction of "oxycholesterol" may simulate the presence of ergosterol in specimens of cholesterol which have been exposed to light or ultra-violet irradiation in presence of air, but is easily differentiated by its absorption band from the ergosterol reaction[1].

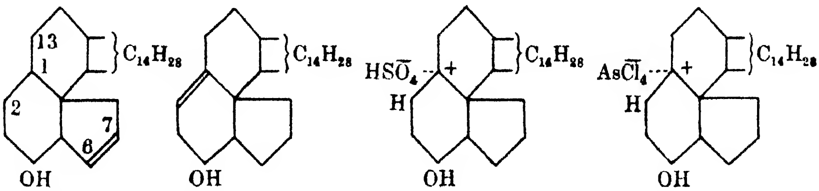
The mechanism of sterol colour reactions.

Various attempts have been made to find an explanation for the numerous sterol colour reactions, which are obviously due to one common factor. Mauthner [1909] considered the presence of double linkages as essential, since the reactions are negative with completely reduced derivatives of cholesterol. In explanation of Liebermann's reaction (acetic anhydride and concentrated H_2SO_4) Wieland and Weil [1913] suggest that the coloured substances are halochromic sulphates of ketones, the latter being produced by the action of acetic anhydride on a reactive double linkage. On the other hand, Whitby [1923] assumes that the sterol colours are produced by the condensation of an aldehydic coupling substance (*e.g.* formaldehyde) with a hydrocarbon (*e.g.* cholesterilene), both of which are presumably formed from the sterol by the condensing agent used (*e.g.* sulphuric acid). There appears to be, however, no evidence for the formation of the postulated aldehydic coupling substance under the conditions of the test, and on other grounds also Whitby's explanation has been criticised [Wokes, 1928].

The reactions described above, which show that an immediate red colour reaction is given only by those sterols or their derivatives which contain the $\Delta^{1,2}$ linkage, suggest a general explanation for the chromogenic properties of sterols. According to this, the primary reaction consists in the isomerisation

[1] The oxidising action of charcoal also gives rise to "oxycholesterol" formation. [Blix and Löwenhielm, 1928.]

of the sterol, under the influence of a strong acid, giving rise to an isomeride containing the $\Delta^{1,2}$ linkage. In these compounds (II) the carbon atom C_1 has become doubly linked. Owing to its position at the junction of two rings, C_1 is assumed to have acquired a property similar to that of the tervalent carbon atom in triphenylmethyl, and to be able, like the latter [Baeyer, 1905, *int. al.*] to form carbonium salts, which are coloured (III and IV).



Cholesterol ⟶ *allo* Cholesterol ⟶ Carbonium salts

I II III IV

This assumption is supported by the fact that the sterol colours are discharged by water or alcohol, and that in their formation strong acids are used (*e.g.* concentrated H_2SO_4 in Salkowski's, Liebermann's, Whitby's, etc. reactions), or generated from the reagent (HCl from $AsCl_3$ in Kahlenberg's reaction). In the latter case, as well as in the trichloroacetic acid reaction, it is significant in this connection that a rapid colour change with sterols other than *allo*cholesterol, etc., requires heat, which also favours isomerisation: a solution of cholesterol in $AsCl_3$ or trichloroacetic acid remains colourless when kept at 0°. The identity of the absorption band in all these reactions with that of *allo*cholesterol is a further indication of the similarity in constitution of the coloured substances formed.

Summary.

1. Ergosterol gives a blue colour reaction with chloral hydrate and with trichloroacetic acid, by means of which it may be detected in the presence of other naturally occurring sterols.

2. An immediate red colour reaction with the above reagents is specific for those sterol derivatives which possess the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage.

3. It is suggested that the primary reaction in all sterol colour reactions consists in the shifting of the double linkage into the $C_{1,2}$ (or $C_{1,13}$) position and the subsequent formation of coloured carbonium salts.

I am indebted to Dr Anderson for specimens of natural dihydrositosterol and γ-sitosterol, to Prof. Drummond for a specimen of *iso*cholesterol from lanolin, and to Messrs Hoffmann-La Roche & Co. and Messrs Boots, Ltd. for zymosterol.

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IX. THE VITAMIN CONTENT OF HONEY.

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Report to the Medical Research Council.

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The experiments recorded in the literature suggest that honey is not a good source of vitamins. Dutcher [1918] tested strained honey from newly-filled combs, obtained while clover and basswood were in bloom, on pigeons and came to the conclusion that the "water-soluble B" content of the product was negligible. Faber [1920] found that daily doses of 4–8·4 cc. of white sage comb honey bought in the open market failed to protect guinea-pigs from scurvy. Hawk, Smith and Bergheim [1921] were also unable to detect vitamin A, B or C in strained honey, as were Scheunert, Schieblich and Schwanebeck [1923] using a centrifuged lime honey from East Prussia, a comb honey with wax from Lüneberger Heide and a honey not of German origin[1]. Great progress has been made in the domain of vitamin research since the above tests were carried out. This communication records the tests for vitamins A, B_1, B_2, C and D on two representative samples of honey as carried out by the more refined methods now available.

Experimental.

One of the samples was a granular honey containing 82·23 % of dry matter and 0·17 % of ash of West Indian origin kindly supplied by Sir Algernon Aspinall. The other sample was an English honey prepared at the Bee Research Institute, Rothamsted, under the supervision of Mr D. Morland, M.A., to whom I should like to express here my indebtedness for the help he extended. The following is Mr Morland's report of the conditions under which the honey was produced.

"The honey was gathered by a stock of Italian bees at Kimpton Hoo, near Welwyn, Herts. A clean super of new frames with wax foundation was given to the colony on July 12th, 1928. At that time the colony had brood in two chambers (British Standard) and had partly filled one super with honey. No queen excluder was used. The weather was favourable throughout the period. The sunshine figures at Rothamsted, which is a few miles from Welwyn, for the period July 12th to July 28th inclusive show a total of 173·8 hours of 'bright sunshine.' White clover was yielding at the time. Much of the neighbourhood is park grassland and the subsoil is chalk. The super was removed

[1] According to the Annual Bureau of Home Economics (1927) two examples of honey examined were found deficient in vitamins A, B, C and D (quoted by Phillips in *Gleanings from Bee Culture*, Jan. 1929).

on July 28th. The hive had increased in weight during this period from 153¼ lb. to 232½ lb. The combs used in the feeding experiment were fully sealed over and the honey appeared to be of excellent quality."

This honey when extracted contained 84·95 % of dry matter and 0·10 % of ash.

In order to extract the English honey from the comb the super was mounted horizontally and the sealings of the cells were removed with a hot knife. The wax and honey of these exposed cells were then removed by means of a spoon and filtered through muslin which retained the wax completely. Just sufficient honey for the day's dosing was extracted daily. The West Indian honey was fed without previous treatment.

Vitamin A.

Vitamin A was estimated by a method based on the Steenbock principle. Young rats weighing 35–40 g. were placed in separate cages on a diet free from the fat-soluble vitamins [Zilva and Miura, 1921]. At the end of 28 days the ordinary cotton-seed oil of the basal diet was replaced by irradiated cotton-seed oil. When the cessation of growth due to the deficiency of vitamin A became apparent, 2 g. of honey were administered daily previous to the delivery of the basal diet. With each test a series of negative controls (on the basal diet alone) and of positive controls receiving 3 drops of cod-liver oil daily were employed. It will be seen from Fig. 1 that the growth curves of the rats which received the honey are similar to those of the negative control animals and consequently a daily dose of 2 g. of honey contained no appreciable quantity of vitamin A.

Vitamins B_1 and B_2.

Vitamins B_1 and B_2 were estimated on the principle described by Chick and Roscoe [1927, 1928], whose help and criticism I wish gratefully to acknowledge. The *L* diet described by these authors was employed as the basal diet. The caseinogen used was B.D.H. "fat-free, vitamin-free casein" and not the specially extracted caseinogen used by them. Six groups of rats were used in the tests and were arranged as follows: group 1, basal diet; group 2, basal diet + 2 g. daily of English honey; group 3, basal diet + 2 g. daily of West Indian honey; group 4, basal diet with 0·4 g. autoclaved yeast + 2 g. English honey daily; group 5, basal diet with 0·4 g. autoclaved yeast + 2 g. West Indian honey daily; and group 6, basal diet and 0·4 g. dried yeast daily. The autoclaved yeast was given to supply vitamin B_2, so that in the event of vitamin B_1 being present in the honey the animals receiving this yeast would have shown growth. The animals in all groups received 3–5 drops of cod-liver oil daily as a source of vitamins A and D. Fig. 2 gives the weight curves of the animals in these groups. With the exception of the positive controls in group 6, the animals of which received both vitamins B_1 and B_2 in the dried yeast

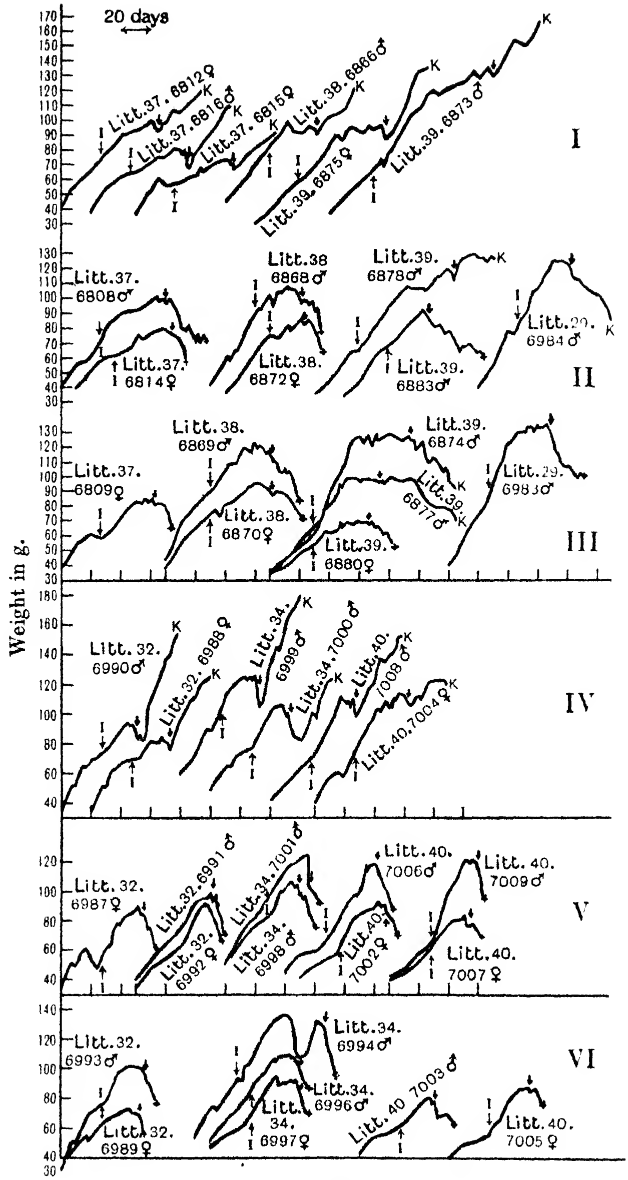

Fig. 1. Test for vitamin A in honey.

I ↓ denotes when irradiated diet first received.

K at end of a graph denotes that animal survived the period of 28 days after giving dose.

↓ denotes when animal began to receive honey.

I Positive controls for West Indian honey.
II West Indian honey.
III Negative controls for West Indian honey.
IV Positive controls for English honey.
V English honey.
VI Negative controls for English honey.

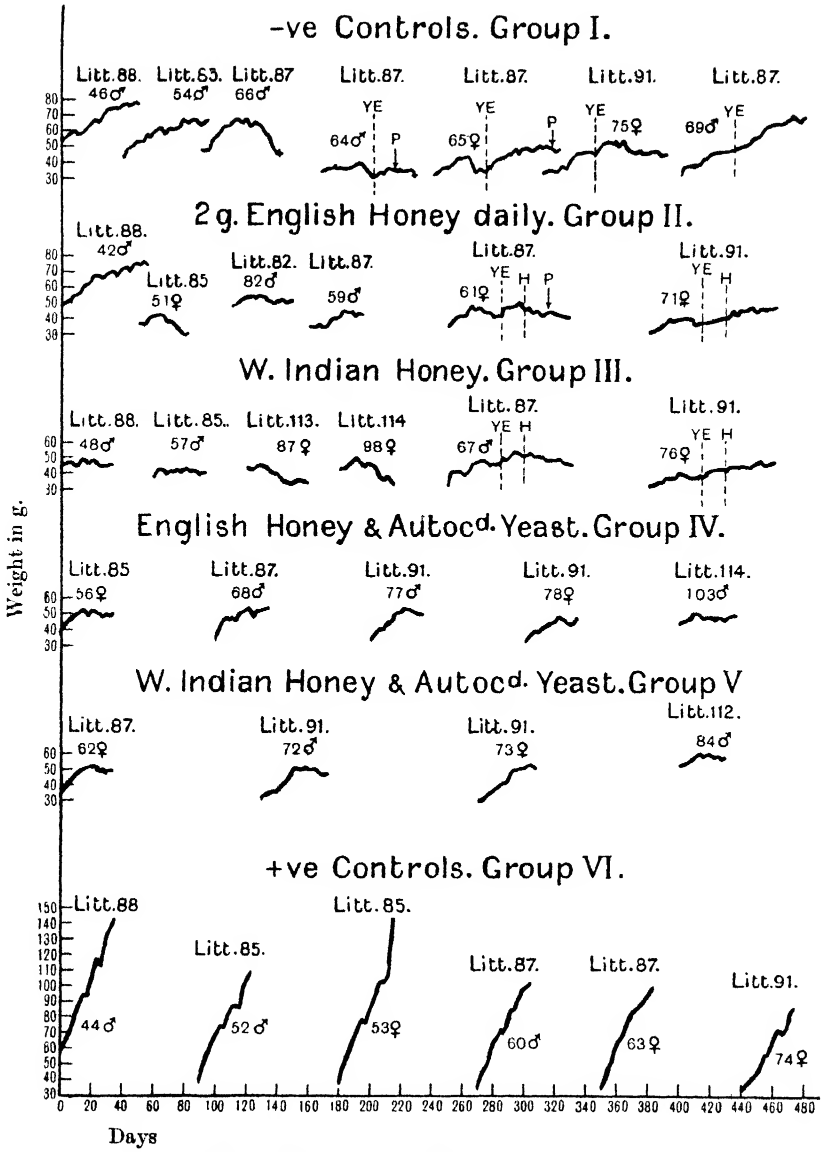

Fig. 2. Test for vitamins B_1 and B_2 in honey.

YE denotes when Peters's extract was first given.

H denotes stoppage of daily dose of honey.

P ↓ denotes definite symptoms of pellagra.

there was little growth. Rats 61, 64, 65, 67, 69, 71, 75 and 76 were given by Miss Chick and Miss Roscoe after the 34th day a daily dose of 0·1 cc. of Peters's antineuritic extract (0·1 cc. daily equal to 0·7 g. yeast) to supply vitamin B_1, and, as will be seen from the weight curves of these animals, no resumption in growth followed this treatment. It may, therefore, be safely assumed that both samples of honey were deficient in vitamins B_1 and B_2.

Vitamin C.

Guinea-pigs subsisting on a scorbutic diet of oats, bran, barley meal, middlings and fish meal, which was offered *ad lib.*, and 40–60 cc. of autoclaved milk, were used in this test. The honey was diluted with about one-third of its weight of water and was administered daily to the animal from the commencement of the experiment. The initial daily dose was 3 g. and it was gradually increased. Certain difficulties were encountered with these tests as the guinea-pigs showed a tendency to succumb to an intestinal disturbance in the early stages of the tests and conclusions had to be drawn from such animals as survived long enough for the development of scurvy. The results are summarised in Table I. It is evident from the figures that although death was somewhat accelerated in certain cases by intestinal disturbance, there was not sufficient antiscorbutic potency in the doses given even to delay the onset of the disease.

Table I. *Test for vitamin C in honey.*

Variety of honey	Ref no. of guinea-pig	Initial wt g.	Maximum wt g.	Final wt g.	No of days alive	Average daily consumption of honey g	Day of 1st appearance of scurvy	P-M. remarks
West Indian	399	315	370	280	27	4·4	15	Died of scurvy
	405	260	295	255	19	5·3	17	Degree of scurvy was such as to indicate that dose was completely negative, though death was due in part to an intestinal disturbance
	406	255	300	220	32	5·1	17	Died of scurvy
	407	260	320	210	28	5·2	17	Died of scurvy
	408	285	287	205	27	5·1	14	Died of scurvy
	409	260	280	250	22	5·4	16	Same condition as no. 405
English comb	410	265	300	240	23	5·3	15	Died of scurvy
	412	285	320	255	22	5·2	14	Scurvy and intestinal disturbance
	413	260	305	200	33	5·1	14	Died of scurvy
	414	260	315	225	29	5·2	14	Died of scurvy
	416	260	260	205	23	4·7	15	Scurvy and intestinal disturbance
	417	260	260	190	25	4·7	18	Died of scurvy
	418	285	290	235	24	5·7	17	Died of scurvy
	419	285	330	255	23	5·9	15	Died of scurvy

Vitamin D.

This vitamin was determined by the Chick, Korenchevsky and Roscoe [1926] method which is based on the comparison of the ratios of the ash to the organic material, other than those substances extractable by hot alcohol and ether, (A/R ratios) of the bones of young rats which have subsisted for about 4 weeks on McCollum's 3143 rickets-producing diet low in phosphorus.

Table II. *Test for vitamin D in honey.*

Rat no.	Initial wt. in g.	Final wt. in g.	Weight of bone g.	% water	% fat	% organic residue	% ash	*A/R*	Average *A/R*
				Positive controls for West Indian honey.					
1 ♀	40	59	0·8407	54·15	4·85	21·54	19·45	0·903	
2 ♂	40	55	0·8139	55·68	6·12	21·26	16·94	0·797	
8 ♂	41	67	1·0236	51·56	6·96	22·70	18·77	0·827	
11 ♂	40	62	0·9800	55·82	6·76	20·79	16·64	0·801	
12 ♂	41	52	0·8590	55·56	5·93	20·96	17·54	0·837	
17 ♀	38	56	0·8048	53·88	4·87	22·23	19·02	0·856	
18 ♂	40	57	0·9040	54·78	6·08	21·79	17·34	0·796	
22 ♂	43	70	0·9396	54·42	6·66	21·09	17·82	0·845	0·833
				2 g. *West Indian honey daily.*					
3 ♀	40	66	0·8829	62·04	4·06	22·64	11·28	0·499	
4 ♂	41	75	1·0293	60·33	5·53	22·04	12·12	0·550	
7 ♂	41	72	1·0670	61·33	4·02	23·41	11·23	0·480	
9 ♀	37	56	0·8331	59·99	5·58	21·80	12·63	0·579	
10 ♂	39	67	0·9225	62·93	4·61	21·55	10·91	0·507	
19 ♀	43	70	0·8752	59·44	3·27	22·40	14·89	0·665	
20 ♂	42	74	1·0725	61·08	4·43	21·44	13·04	0·608	
21 ♀	43	75	0·9456	62·34	3·24	23·46	10·96	0·467	0·544
				Negative controls for West Indian honey.					
5 ♀	41	72	0·9447	63·67	3·12	22·89	10·31	0·451	
6 ♂	41	76	0·9438	63·44	3·78	22·68	10·08	0·445	
13 ♂	41	65	0·9187	63·53	2·78	22·52	11·18	0·497	
14 ♂	40	63	0·9475	62·70	4·98	22·07	10·27	0·465	
15 ♀	43	77	0·9305	61·64	3·63	22·26	12·47	0·560	
16 ♂	42	68	0·9556	63·83	3·33	22·29	10·54	0·473	
23 ♂	44	76	0·9610	59·60	5·00	21·61	13·79	0·638	0·504
				Positive controls for English comb honey.					
24 ♀	45	59	0·7873	55·49	7·06	21·42	16·02	0·747	
25 ♂	45	57	0·8233	50·79	9·97	20·91	18·31	0·876	
26 ♀	45	57	0·8309	52·36	8·55	21·37	17·72	0·827	
36 ♂	42	69	0·7506	53·26	6·32	21·54	18·87	0·878	
37 ♀	39	60	0·8693	54·80	7·82	21·20	16·18	0·763	
38 ♂	40	67	0·9292	53·30	8·50	21·13	17·07	0·807	0·812
				2 g. *English comb honey daily.*					
27 ♂	43	71	0·9546	63·78	5·97	20·84	9·42	0·452	
28 ♀	43	68	0·8510	63·62	3·73	22·84	9·81	0·429	
29 ♂	50	78	0·9449	62·14	4·89	22·84	10·06	0·440	
39 ♀	35	52	0·6489	63·33	4·41	23·93	8·32	0·347	
40 ♂	39	58	0·7509	63·98	4·07	23·03	8·92	0·386	
41 ♀	41	58	0·7524	64·49	3·07	24·64	7·80	0·317	0·395
				Negative controls for English comb honey.					
30 ♀	43	70	0·8759	62·66	4·69	22·32	10·35	0·464	
31 ♂	45	79	0·9889	62·62	4·49	22·62	10·26	0·454	
32 ♂	48	75	0·9362	61·94	4·43	22·05	11·57	0·525	
33 ♀	39	54	0·7320	62·91	4·06	24·51	8·52	0·348	
34 ♂	41	63	0·8126	65·88	3·62	22·92	7·60	0·333	
35 ♀	38	59	0·7987	64·91	3·42	23·58	8·09	0·343	0·411

The animals received a daily dose of 2 g. of each of the honeys preparea as above. With each honey a group of negative control animals on the basal diet only and a positive group which received 3 drops of cod-liver oil in addition were employed. As will be seen from Table II the differences between the *A/R* ratio of the rats receiving the honeys and those of the corresponding negative control animals are of an order which falls within the limits of accuracy of the method. It may be safely assumed that both the honeys were inactive in the doses employed in the test.

Conclusion.

Tests carried out on a fresh English sample of comb honey and on a West Indian honey show that both samples are deficient in vitamins A, B_1, B_2, C and D. These results are in consonance with those obtained by other workers, which show that honey is not a source for these vitamins and that this deficiency is not due to deterioration consequent on treatment or storage.

My thanks are due to Dr S. S. Zilva, under whose direction the investigation was carried out, for help and criticism.

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X. THE RELATION OF THE GROWTH OF CERTAIN MICRO-ORGANISMS TO THE COMPOSITION OF THE MEDIUM.

IV. THE ADDITION OF MANNITOL.

By VERA READER.

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(*Received January 1st, 1929.*)

In a paper by Orr-Ewing and Reader [1928], a technique was described for using the growth-promoting power of yeast concentrates upon *Streptothrix corallinus* as a test for the antineuritic vitamin. In this technique the yeast concentrate under test was put up in three dilutious, viz. 1/20, 1/400, 1/8000 in equal volumes of medium (20 cc.), and the resulting streptothrix growth compared with that in a series of standard flasks containing similar dilutions and intermediate dilutions of a concentrate of known potency. The result was expressed in terms of pigeon doses. When testing the purer antineuritic concentrates agreement with the corresponding standards was obtained in all three dilutions, *e.g.* 80, 70, and 75 doses in a given test sample. However, certain "anomalous results" appeared with the less pure concentrates; the tests came out thus, 500, 70, 75; *i.e.* there appeared to be relatively more growth-promoting activity in the higher concentrations of vitamin than in the lower. In view of this discrepancy, further work was undertaken to elucidate the phenomenon.

It has often been noted that the upper limit of concentration necessary for maximum growth is reached when approximately 1/80 of a pigeon-day-dose has been added to 20 cc. of the medium. Further addition of glucose or of vitamin concentrate at this point does not induce further growth. Now the anomalous result always occurred when the yeast concentrate was comparatively impure, and this suggested that the abnormally high results with the larger additions of vitamin were due to some added source of food for the organism. A few preliminary experiments done in 1924 had seemed to suggest that some specific carbohydrate or polyhydric alcohol might give greater growth in a given time at a given temperature than that obtained with glucose. Further, from earlier published work [Reader, 1927] it was known that "the following substances may replace glucose as a source of carbon in the medium, without loss of efficiency: glycerol, mannitol, arabinose, lactate, citrate, pyruvate, and glycogen." As these earlier experiments were carried out in a medium of pure salts + carbon compound, it was now decided to test their effect as additional substances to the seemingly complete medium.

Experimental.

The technique differs from that described in Part III [Reader, 1928] in one particular only, that of method of estimation. Since all the experiments in this section are concerned with the requirements for maximal growth and not with minimal dilutions for growth, it was possible to estimate the amount of growth by direct weighing. The contents of three small similar conical flasks (20 cc. fluid) were filtered through the same small, hard, dried and weighed filter-paper. The whole was then dried in a $CaCl_2$ desiccator to constant weight. Since the variation in twenty similar papers was not greater than $\pm$ 5 %, this method was considered to be of sufficient accuracy. Growth was estimated at the end of 6 days, unless otherwise stated. Conditions of temperature are quoted at the beginning of each experiment.

Exp. 1. The amounts of synthetic medium, glucose, and torulin (vitamin) extract were kept constant throughout seven sets of three flasks, while 0·5 % of the various compounds under test was added severally to each of the sets (Table I).

Table I.

T = 1/100 unit dose of torulin in 20 cc. fluid.
Temp. 31° for 2 days followed by 15° for 4 days.

Set	Medium	Total weight from three flasks g.
I	Salt-sugar medium + T	0·025
II	,, ,, + T	0·025
III	,, ,, + T + arabinose	0·024
IV	,, ,, + T + glycogen	0·021
V	,, ,, + T + glycerol	0·041
VI	,, ,, + T + citrate	0·065
VII	,, ,, + T + mannitol	0·144

It will be noted that the six-fold increase of growth with mannitol is of the order of the divergence of the "anomalous results" from the normal. Consequently Sets I and VII were repeated, dilutions being made as in the test cases described in the paper by Orr-Ewing and Reader [1928], in each case the mannitol being diluted with the torulin.

When torulin and mannitol were diluted 400 times, *i.e.* as in the third flask of the test, growth was equivalent to that with torulin without mannitol, hence a true case of "anomalous result."

Exp. 2. The growth obtained in Set VII of exp. 1 was so prolific that it was decided to extract the dried organisms with ether to find out whether the increased weight was due to increased fat production rather than to an increase in the number of organisms (see Table II).

Table II.

	Weight of organism g.		% fat-soluble material
I	Growth without mannitol	0·045	6·0
II	Growth with mannitol	0·144	6·1
III	,, ,,	0·142	6·0

From these figures it was concluded that mannitol had not increased the fat production.

Exp. 3. The mannitol used in the above experiments was as supplied by Baird and Tatlock and was supposed to be pure. However, considering the earlier experience with traces of accessory factor present as impurity in glucose [Reader, 1928] an attempt was now made to purify the mannitol from any such factor. In the first place the mannitol was tested before and after extraction with charcoal in neutral solution, but this was found to have no effect, the respective growths being 0·135, 0·133 and 0·132 for the original mannitol and after one and two treatments with charcoal.

While developing a technique for the extraction of the antineuritic vitamin Kinnersley and Peters [1925] found that control of hydrogen ion concentration was an important factor in the removal of such accessory factors by charcoal adsorption. A more exhaustive attempt at removal on charcoal at various hydrogen ion concentrations was now undertaken, but again without success (Table III).

Table III.

T = 1/100 unit dose of torulin in 20 cc. fluid.
Temp. 31° for 2 days followed by 15° for 4 days.

Set	Medium	Weight of growth g.
I	Salt-sugar medium $+T$	0·033
II	,, ,, $+T+M$ (crude, 0·1 g. in 20 cc.)	0·140
III	,, ,, $+T+M$ (purified charcoal at $p_H=2$)	0·137
IV	,, ,, $+T+M$ (purified charcoal at $p_H=5$)	0·138
V	,, ,, $+T+M$ (purified charcoal at $p_H=7$)	0·135
VI	,, ,, $+T+M$ (purified charcoal at $p_H=9$)	0·139
VII	,, ,, $+T+M$ (hydrolysed dilute HCl for 30 mins.)	0·127
VIII	,, ,, $+T+M$ (hydrolysed dilute NaOH for 30 mins.)	0·121

As the results show, no change was produced by these various modes of treatment. Additional proof that the growth-promoting property is due to the presence of the mannitol itself, and not to an impurity, was obtained by reducing a sample of mannose which had been previously tested and found to have no enhancing effect on the growth (Table IV). The reduction was carried out according to the directions of Fischer [1890], using Na amalgam in alkaline solution with rapid mechanical stirring for 12 hours.

Table IV.

Set	Medium	Growth g
I	Salt-sugar medium $+T$	0·033
II	,, ,, $+T+$ mannose (0·5 %)	0·028
III	,, ,, $+T+$ mannitol (0·5 %) (from mannose)	0·090

It was now decided to test other available polyhydric alcohols. Similar experiments were designed with dulcitol, sorbitol, glycerol and inositol (Table V). From exp. 5, Table V it may be seen that the effect with none of them was of the order of that obtained with mannitol, *i.e.* the enhancement with mannitol appears to be a specific one for this organism.

Table V.

Set	Medium	Weight of growth g.
I	Salt-sugar medium $+T$	0·033
II	,, ,, $+T+$mannitol	0·140
III	,, ,, $+T+$dulcitol	0·065
IV	,, ,, $+T+$sorbitol	0·045
V	,, ,, $+T+$inositol	0·057
VI	,, ,, $+T+$glycerol	0·058

With regard to the phenomenon of the optimum concentration of the growth factor referred to in the introduction (p. 61) it now seemed possible that, in the presence of mannitol, any excess of growth-promoting factor supplied might be used up. In the following experiments the amounts of mannitol and glucose were kept constant throughout, while the torulin concentrate was varied from 1/20 to 8/20 day-dose per 20 cc. The results are shown in Table VI. From these, it may be concluded that there is a definite upper limit of torulin concentrate for maximum growth even in the presence of mannitol, but that that upper limit is much greater than with glucose alone, as is also the maximum growth obtained. It is easily seen from the curves for the four experiments cited that in each case the addition of mannitol has led to an abnormally high value for the maximum growth. Therefore, with the highest concentration of torulin each case is a true "anomalous result."

Table VI.

(All flasks contain 20 cc. inorganic salt-sugar medium.)

$G=0{\cdot}5\ \%$ glucose. $M=0{\cdot}5\ \%$ mannitol. $T=1/20$ unit dose torulin concentrate.

Exp. 6. Temperature 3 days at 33° then 6 days at 16–20°.
Exp. 7. ,, 3 ,, 33° ,, 6 ,, 20–25°
Exp. 8. ,, 6 ,, 33°.
Exp. 9. ,, 6 ,, 33° ,, 3 ,, 16–20°.

	Medium	g. growth Exp. 6	Exp. 7	Exp. 8	Exp. 9
1.	$G+T$	0·056	0·060	0·057	0·061
2.	$G+4T$	0·056	0·061	0·050	0·065
3.	$G+8T$	0·056	0·060	0·059	0·064
4.	$G+M+T$	0·073	—	0·138	0·090
5.	$G+M+2T$	0·090	0·186	0·151	0·113
6.	$G+M+4T$	0·084	0·187	0·161	0·143
7.	$G+M+8T$	0·111	0·180	0·154	0·171

The variations in the results ($G+M+xT$) are due to variation in the average room temperature during the time after the cultures had been removed from the incubator. This was very puzzling at first, until it was realised that not only the torulin efficiency, but also the temperature coefficient had been appreciably altered by the addition of mannitol. Thus, in exp. 6 the temperature on the latter days varied from 16° to 20° and in exp. 7 from 20° to 25°. The difference between the results is greater than in exps. 8 and 9 where the change from the incubator temperature to that of the room occurred at a later stage in the life cycle of the organism [see Reader, 1926].

Much further work is needed upon this point, more especially as the issue

is complicated by the fact that the organism under observation is a *Streptothrix*, that is, it is continually segmenting, and the rate of the segmentation is also varied by slight changes in temperature.

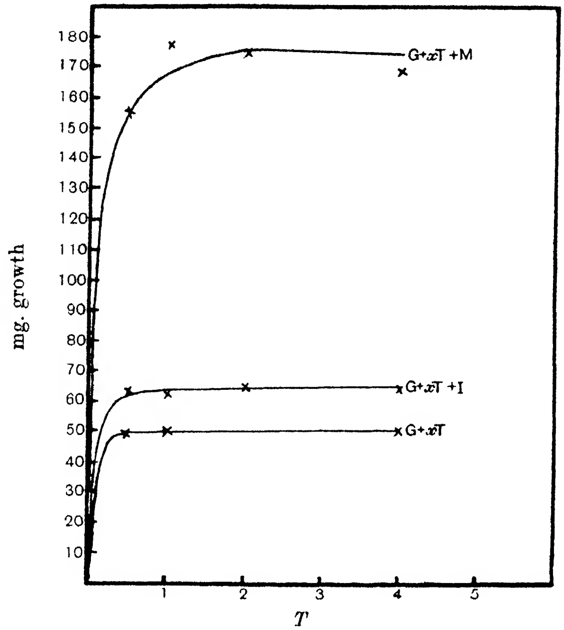

Fig. 1. Weight of organisms in 14 days, *i.e.* after growth is complete.
(M = 0·5 % mannitol; I = 0·5 % inositol; G = 0·5 % glucose; T = 1/20 unit dose of torulin.)

Table VII.

Temperature 7 days at 33°, then 7 days at 20°

I = 0·5 % inositol. M = 0·5 % mannitol. T = 1/20 unit dose torulin concentrate.

	Medium	Weight of growth in 14 days g.
1.	$G+T$	0·050
2.	$G+2T$	0·051
3.	$G+M+1/10T$	0·070
4.	$G+10M+\frac{1}{2}T$	0·160
5.	$G+M+\frac{1}{2}T$	0·155
6.	$G+1/10M+\frac{1}{2}T$	0·154
7.	$G+10M+T$	0·170
8.	$G+M+T$	0·179
9.	$G+1/10M+T$	0·175
10.	$G+10M+2T$	0·169
11.	$G+M+2T$	0·175
12.	$G+1/10M+2T$	0·178
13.	$G+10M+4T$	0·174
14.	$G+M+4T$	0·165
15.	$G+1/10M+4T$	0·170
16.	$G+I+\frac{1}{2}T$	0·064
17.	$G+I+T$	0·062
18.	$G+I+2T$	0·067
19.	$G+\frac{1}{2}I+T$	0·057
20.	$G+I+4T$	0·063

A final, and perhaps more conclusive experiment was carried out (see Fig. 1). In this the weighings were taken at the end of 14 days. The curve for inositol is added for comparison. The results are given in Table VII. As the growth was independent of concentration of mannitol, at least within these limits, only one curve is drawn ($G + xT + M$) (Fig. 1).

Thus it may be seen that even with 1/200 of a unit dose (1/10 T) the growth with mannitol is in excess of the maximum growth with glucose alone, and also that the maximum growth with mannitol is not reached until 1/20 of a unit dose is supplied in 20 cc. of medium. From this table it is obvious that in the presence of mannitol a true "anomalous result" is artificially reproduced.

Discussion.

Whether the results obtained by Orr-Ewing and Reader with crude torulin extracts were due to the presence of mannitol in the watery extracts of yeast has not yet been proved. Indeed it would appear that the prolonged extractions and purification of yeast extract necessary to show the presence of mannitol would be unlikely to meet with success as the work is complicated by the presence of inositol [see Eastcott, 1928] and possibly other polyhydric alcohols of very similar properties.

At this point it is interesting to consider the parallel case reported by Eastcott [1928]. She claims to have isolated "bios I" [see Lucas, 1924] for yeast, and to have identified this substance with inactive inositol, the "phaseomannite" of Vohl [1856]. However the degree of stimulation reported in her experiments is not greater than that recorded in this paper for the action of mannitol on the growth of *S. corallinus*. Thus, from her experiments, it may be seen that in 48 hours, in solutions containing salts, sugar and "bios II," 113 cells of yeast were produced, while with salts, sugar, crude "bios II" and inositol, some 365 cells resulted from the same size of inoculation. This increase is of the same order as that shown in Tables VI and VII of this paper. Certainly I do not consider that the mannitol effect may be explained as due to a true "growth-promoting factor," but rather as a specific source of food-supply and would suggest a similar interpretation of the results of Eastcott.

Summary.

Further work on the cultural requirements of *S. corallinus* is reported.

1. The "anomalous results" obtained by Orr-Ewing and Reader when crude watery extracts of yeast were added to the culture medium, can be artificially reproduced by adding mannitol to the purer extracts.

2. The increased weight of bacteria grown in the presence of mannitol was not due to increased fat production.

3. Within the limits of these experiments, the amount of growth was independent of the concentration of the mannitol.

4. Exhaustive treatment with charcoal at various hydrogen ion concentrations and subsequent recrystallisation failed to remove any growth-promoting factor from the mannitol.

5. A sample of mannitol was prepared from mannose which had previously been shown to have no growth-promoting effect. This mannitol gave an enhancement of growth equal to that obtained with mannitol from natural sources as usually supplied for laboratory purposes.

6. It is not possible to substitute dulcitol, sorbitol, glycerol, or inositol for the mannitol.

It is concluded that the effects obtained are due to either the whole or part of the mannitol molecule acting as a specific source of food-supply rather than as an additive "growth-promoting factor."

My thanks are due to Prof. R. A. Peters for his continued criticism and advice, and to the Medical Research Council for a grant.

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XI. THE DIALURIC ACID-ALLOXAN EQUILIBRIUM.

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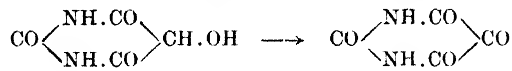
In acid solution, dialuric acid and alloxan may readily be converted into one another. The change is one involving two equivalents of hydrogen:



The addition of one equivalent of a reducing agent to alloxan or of one equivalent of an oxidising agent to dialuric acid leads, on the other hand, to the separation of the sparingly soluble substance, alloxantin. The latter is also formed when solutions of these two reactants are mixed in equivalent proportions, and it has therefore been natural to regard it as a molecular compound comparable to the quinhydrones and meriquinones. It is generally believed that alloxantin is extensively dissociated into its two components in aqueous solution.

Our interest in the above system originated in the demonstration by Biilmann and Lund [1923] that solutions of alloxantin developed constant and reproducible potentials at a platinum electrode in the absence of oxygen. Their observations, which were restricted to solutions of alloxantin in 0·1 and 0·02 M sulphuric acid, indicated that the observed potentials (E_h) were related to the hydrogen ion activity by the equation[1]

$$E_h = E_0 - \frac{RT}{F} \ln [\mathrm{H}^+],$$

E_0 being a constant with a value of 0·3698 at 18° and of 0·3664 at 25°. Assuming that alloxantin dissociated into equivalent amounts of dialuric acid and alloxan, Biilmann and Lund plausibly attributed the potentials of solutions of alloxantin to the reversible oxidation-reduction system

$$\text{dialuric acid} = \text{alloxan} + 2e.$$

The studies which will be reported in this paper have had as their object the quantitative proof of this assumption, the extension of the electrode data

[1] $[\mathrm{H}^+]$ refers throughout to the hydrogen ion activity measured electrometrically. $[a]$, $[b]$ and $[m]$ of the electrode equations are actually molar concentrations, the assumption being made that these are sensibly equal to the activities at the experimental concentrations employed.

to cover the whole significant p_H range, and the examination of the effects upon the electrode behaviour of the system of the formation of alloxantin. It early became apparent that the system was further complicated by the fact that the oxidant was subject to an irreversible change at a rate which was a function of the hydroxyl ion concentration, and it therefore became necessary to relate the kinetics of this change to the electromotive behaviour of the system.

EXPERIMENTAL.

The dialuric acid, alloxan and alloxantin which were employed were prepared from uric acid in the usual way, and their purity established by determinations of nitrogen, base-binding capacity, and (in the cases of dialuric acid and of alloxantin) their iodine-reducing capacities.

In order to comprehend the relations of the equilibrium potentials to $[H^+]$, it was desirable to have independent determinations of those ionisation constants of the reactants which were effective within the experimental p_H range. The acidic constant of dialuric acid was measured with precision by means of a hydrogen electrode titration curve in the usual manner. This titration betrayed no other groups having constants of significant values. The case of alloxan was more difficult. Its ease of hydrogenation renders the hydrogen electrode method inadmissible. Moreover, the degree of ionisation of alloxan determined experimentally appears to change with time. Woods [1906] found that the conductivity of solutions of alloxan increased steadily, and Biilmann and Bentzon [1918] observed that there is also an increase in their $[H^+]$ as determined by colorimetric methods. These effects are, most probably, due to the isomeric change to alloxanic acid which readily takes place under the influence of hydroxyl ions. In an attempt to construct a titration curve by means of colorimetric p_H determinations, we observed this change in p_H in all our mixtures of alloxan and sodium hydroxide. For any given mixture, however, $d\,(p_H)$ is approximately a linear function of the time. It was, therefore, possible to extrapolate to zero time the p_H values of such mixtures (where α varied from 0·09 to 0·58). The section of the titration curve thus constructed led to a quite consistent series of values for the first (acidic) constant of alloxan. It will be shown, later, that the electrode data indicate the presence of a second acidic constant of significant magnitude as well as a very weak basic group. The constant of the latter seems to be too small for direct measurement [cf. Biilmann and Bentzon, 1918] while the demonstration of the former was not attempted because of the instability of alkaline solutions.

The technique employed in the determination of the electrode potentials has already been outlined [Cannan, 1926]. Both dialuric acid and alloxantin proved to be very prone to autoxidation. It was necessary, therefore, to employ all the usual precautions for the total exclusion of oxygen during the preparation and manipulation of solutions of the reactants. Electrode potential titration curves for the titration of dialuric acid (0·002 M) in 0·05 M buffer

solutions [Clark, 1928] with 0·02 M potassium ferricyanide or iodine were obtained at several p_H values between 1 and 5. Both platinum and gold-plated electrodes were employed and generally gave concordant readings. The former, however, were frequently sluggish in reaction, whereas the gold-plated electrodes consistently adjusted themselves to changes in the system within two or three minutes. The main part of the curves traced by titrating with iodine and with ferricyanide were identical, but iodine sometimes gave distortions of the initial and terminal portions of the curves, so rendering less precise the determination of end-points. For the detailed analysis of the data, therefore, we have restricted ourselves to the ferricyanide titrations and the gold-plated electrodes. All electrometric observations were made on solutions maintained at 30° in a thermostat.

The titration data for the relation of the electrode potentials to [H^+] were further elaborated by observations of the equilibrium potentials of 0·001 M solutions of alloxantin in a series of 0·05 M buffer solutions with p_H values intervening between those of the complete titration curves.

On the alkaline side of p_H 5 steady potentials were never attained, either in solutions of alloxantin or in mixtures of alloxan and dialuric acid. Instead, linear drifts towards increasing negative values were consistently observed. It was, therefore, not possible to conduct titrations in this range, but it proved to be possible to obtain consistent values for the equilibrium potentials of alloxantin by extrapolating to zero time the potential-time drifts. Theoretical justification for this procedure will be given later.

The p_H values of all systems studied were determined directly by means of the hydrogen electrode.

Results.

In Table I are assembled the data for a typical titration curve at p_H 1. At constant p_H the electrode equation for the system dialuric acid-alloxan is

$$E_h = E_0' - 0{\cdot}03006 \log \frac{[b]}{[a]}, \qquad \text{......(1)}$$

E_0' being a constant, and [b] and [a] being the respective concentrations of dialuric acid and alloxan. The constancy of E_0' when this equation is applied to the data is satisfactory evidence that the potentials are related to a simple reversible system involving two equivalents of hydrogen.

The first three values for E_0' in Table II have been derived from complete titrations in this way. The remaining values are equilibrium potentials of solutions of alloxantin derived by direct observation or by extrapolation. All these and others are displayed in Fig. 1, which sufficiently demonstrates that the alloxantin potentials agree well with the values of E_0' obtained by titration, and that these are plausibly extended into the alkaline ranges by the values obtained by extrapolation of the time-potential drifts. In other words, the potentials of alloxantin may properly be attributed to the presence in solution of equivalent amounts of dialuric acid and alloxan.

Table I.

Titration of 50 cc. of 0·00175 M dialuric acid with 0·02024 M potassium ferricyanide in 0·1 M HCl. Temperature 30°. p_H 1·043.

Titre	$0{\cdot}03006 \log \frac{[b]}{[a]}$	E_h	E_0'
0·15 cc.	+0·0525	+0·2505	[+0·3030]
0·23	0·0468	0·2576	[0·3044]
0·35	0·0412	0·2620	[0·3032]
0·55	0·0350	0·2669	0·3019
0·85	0·0288	0·2730	0·3018
1·15	0·0243	0·2775	0·3018
1·65	0·0187	0·2833	0·3020
2·35	0·0127	0·2888	0·3015
3·15	0·0070	0·2948	0·3018
4·15	+0·0008	0·3011	0·3019
5·15	−0·0054	0·3070	0·3016
6·15	0·0123	0·3138	0·3015
6·95	0·0192	0·3211	0·3019
7·65	0·0279	0·3297	0·3018
8·15	0·0393	0·3415	0·3022
8·35	0·0487	0·3503	0·3016
8·55 (end-point)			
			Mean 0·30185±0·00035

Table II.

Relation of E_0' to p_H. $E_0 = +0{\cdot}3640$.

p_H	E_0' Found	E_0' Calculated	Difference	Method
1·043	+0·3018	+0·3015	0·0003	Titration of 0·002 M dialuric acid
2·943	0·1976	0·1979	0·0003	
4·884	0·1320	0·1322	0·0002	
2·127	0·2388	0·2385	0·0003	Equilibrium potentials of 0·001 M alloxantin
2·549	0·2143	0·2164	0·0021	
3·153	0·1879	0·1891	0·0012	
3·757	0·1668	0·1675	0·0007	
4·155	0·1549	0·1546	0·0003	
4·565	0·1426	0·1420	0·0006	
4·965	0·1294	0·1297	0·0003	
5·420	0·1155	0·1158	0·0003	
5·885	0·1009	0·1014	0·0005	
6·555	0·0789	0·0790	0·0001	Extrapolated potentials for 0·001 M alloxantin
7·027	0·0602	0·0611	0·0009	
7·494	0·036	0·039	0·003	
8·025	+0·011	+0·011	—	
9·007	−0·052	−0·048	0·004	
9·955	0·112	0·112	—	
10·370	0·146	0·144	0·002	
11·055	0·205	0·202	0·003	
11·955	0·274	0·282	0·008	

Table III.

Dissociation constants (uncorrected for activity). Temperature 30°.

	Group	K′	$p_{K'}$	Method
Dialuric acid	1st acidic (K_r')	$1{\cdot}48 \times 10^{-3}$	2·83	Hydrogen electrode
	2nd „	Negligible	>11	$E_0' : p_H$ relation
	3rd „	„	>11	„
Alloxan	1st acidic (K_1')	$6{\cdot}31 \times 10^{-8}$	7·20	Colorimetric
	2nd „ (K_2')	$1{\cdot}0 \times 10^{-10}$	10·0	$E_0' : p_H$ relation
	Basic	Negligible	<1	„

Fig. 1 betrays a system more complicated than was expected. The "0·09 slope" exhibited at extreme alkalinity together with the fact that both dialuric acid and alloxan have acid properties suggest that the type of system which best fits the structural relations of the reactants is

$$\begin{bmatrix}\text{reductant}\\ \text{anion}\end{bmatrix}^{---} \rightleftharpoons \begin{bmatrix}\text{oxidant}\\ \text{ampholyte}\\ \text{anion}\end{bmatrix}^{+--} + 2e$$

The values to be assigned to the dissociation constants of the various ionisable hydrogen atoms are given in Table III. The strong acid group of dialuric acid must be assigned to the carbon atom carrying the hydroxyl group, and, according to the scheme, this atom must, therefore, assume basic properties on oxidation. Indeed, alloxan may, as has been suggested by Richter [1911], be

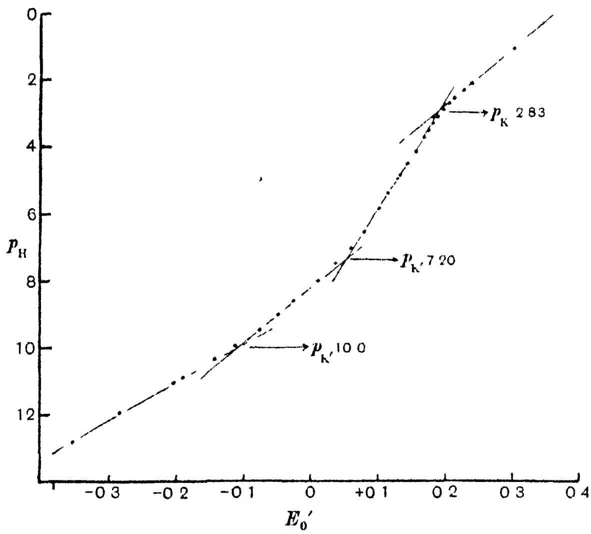
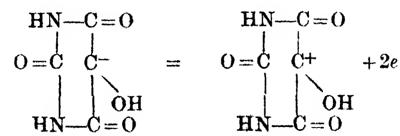


Fig. 1. E_0' : p_H curve

an oxonium base, though our allocation of constants would give it only negligible basic properties. The remaining acidic hydrogen atoms of the two reactants may, plausibly, be identified with the hydrogen atoms attached to nitrogen:



dialuric anion alloxan cation

With the assumptions of Table III, the rational equation relating E_0' to p_H may be simplified to

$$E_0' = E_0 + 0{\cdot}03006 \log \frac{[H^+]^3[K_r' + H^+]}{[K_1' + H^+][K_2' + H^+]}. \qquad \ldots\ldots(2)$$

Adopting a value of $+ 0{\cdot}3640$ for the constant E_0 there may be calculated the theoretical values of E_0' found in Table II. In acid solutions, where the

experimental values are true equilibrium potentials, the agreement is within the experimental error. In neutral and alkaline solutions, where the quoted values are extrapolated from the time course of a drifting potential, greater latitude must be allowed. When it is pointed out that the significant range of potential drift is less than 0·05 v. and that the observed rates rose to values as great as 0·02 v. per minute, it is apparent that precise extrapolation is quite impossible.

It is well known that the initial equilibrium of an electrode with an active system is frequently much slower than subsequent adjustments to changes in that system. This was so in the present case. The observations on solutions of alloxantin in which the potential drift was rapid were, therefore, made by adding 4 cc. of 0·01 M alloxantin to 30 cc. of a deaerated buffer of acid p_H, and then, after about fifteen minutes, adding the necessary amount (about 10 cc.) of sodium hydroxide to bring the solution to the desired p_H. The exact p_H value of the solution was determined by the hydrogen electrode at the conclusion of the experiment. The drift of potential was measured from the time of addition of alkali. Using this method, it was possible by observations every half minute to establish, fairly definitely, the linear drift even in the cases where the change was most rapid. There can be little doubt that the data for the more alkaline solutions do establish—in spite of their crudeness—the reality of the two inflexions in the $E_0' : p_H$ curve at p_H 7·2 and 10 respectively (Fig. 1).

The value we have found for E_0 at 30° is quite consistent with those obtained by Biilmann and Lund at 25° and at 18°. The three values may be accurately combined in the expression

$$E_0 = 0{\cdot}3784 - 0{\cdot}00048\,t.$$

The degree of dissociation of alloxantin.

In Table I the tacit assumption was made that the active mass of dialuric acid at each stage in the titration was equal to the fraction of the initial concentration which remained unoxidised. The active mass of alloxan was, likewise, identified with the fraction of the initial concentration of dialuric acid which had suffered oxidation. If, however, significant amounts of the two reactants associate to form alloxantin then these assumptions become unsound. Under such conditions it may be shown that the correct equation for a titration at constant p_H is

$$E_h = E_0' - \frac{RT}{2F} \ln \frac{[S_r] - [m]}{[S_o] - [m]}, \qquad \ldots\ldots(3)$$

where $[S_r]$ and $[S_o]$ are the concentrations of total reductant and oxidant respectively, and $[m]$ is the concentration of alloxantin. The latter is defined by

$$K = \frac{[m]}{[a]\,[b]}. \qquad \ldots\ldots(4)$$

Now K has been determined by Biilmann and Bentzon from studies upon the solubility of alloxantin in the presence of varying concentrations of added

alloxan. They arrived at a mean value of 39. This would indicate that the degree of association in 0·001 M solution would not be sufficient to give values of $[m]$ significant for equation (3). That we were able to ignore this quantity in Table I agrees with this conclusion. At higher concentrations of reactants, however, the degree of association should lead to a distinct distortion of the normal curve. This we found to be the case in the titration of 0·01 M dialuric acid at p_H 1 (Table IV). From the titres may be calculated $[S_r]$ and $[S_0]$ in the usual way and thence equation (3) may be solved for $[m]$. Finally K may be calculated from (4). In view of the fact that the method is not well adapted to the accurate measurement of such an association constant, the values derived in Table IV must be held to be in satisfactory agreement.

Table IV.

Titration of 0·00897 M dialuric acid with 0·0508 M potassium ferricyanide

$p_H = 1{\cdot}080$. $E_0' = +0{\cdot}2990$.

Titre	E_h	$S_0 \times 10^6$	$S_r \times 10^6$	$m \times 10^6$	K
0·41	+0·2572	413·5	8483	71·8	25
0·71	0·2655	711·6	8128	94·5	19
1·01	0·2709	1006	7789	114·7	17
1·51	0·2773	1490	7214	150·0	16
2·21	0·2836	2151	6434	249 5	21
3·01	0·2899	2886	5569	223·3	16
4·01	0·2964	3773	4527	345·5	24
5·01	0·3030	4629	3520	432·0	32
5·51	0·3061	5046	3031	243·2	18
6·01	0·3095	5454	2549	199 2	16
6·81	0·3159	6091	1799	179·0	19
7·51	0·3231	6633	1162	136·6	21
8·01	0·3306	7016	712	97·0	23
8·31	0·3375	7241	445	69·7	26
8·82 (end-point)					
					Mean 21

Table V.

Velocity of decomposition of alloxan. Temperature 30°.

p_H	Change of E_h per min.	$k = \frac{1}{t} \ln \frac{a}{a-x}$
4·97	−0·000034	0·0026
5·31	−0·00010	0·0077
5·74	−0·00016	0·012
6·00	−0·00037	0·028
6·01	−0·00080	0·061
6·56	−0·0027	0·21
7·03	−0·012	0·92
7·49	−0·018	1·4
7·99	−0·020	1·5
8·03	−0·020	1·5
8·80	−0·016	1·2
9·00	−0·020	1·5
9·01	−0·020	1·5
9·79	−0·016	1·2
9·96	−0·014	1·1
10·37	−0·018	1·4
11·06	−0·016	1·2

Our mean value will be seen to be about half that of Biilmann and Bentzon. Since the p_H of the saturated solutions with which these authors worked would be about 2·4, the discrepancy is much too small to be due to a primary

effect of hydrogen ion activity. More plausibly it may be attributed to the difference in temperature, and in ionic strength, of the solutions in the two studies. We may conclude as a first approximation that K is independent of $[H^+]$ in this range.

The rate of isomeric change of alloxan.

There is little doubt that the drifts of potential of solutions of alloxantin towards more negative values are due to a slow loss of active oxidant. Since they are accelerated by hydroxyl ions and parallel the increases in acidity and conductivity of solutions of alloxan, it is most probable that they are due to the isomeric change of the latter to alloxanic acid. Assuming, therefore, the change to be monomolecular, we have for a buffered solution of alloxantin

$$kt = \ln \frac{a}{a-x},$$

where a is the initial concentration of alloxan and $a - x$ the concentration at time t. At this time, also,

$$E_0' - E_h = \frac{RT}{2F} \ln \frac{a}{a-x}.$$

Hence
$$E_0' - E_h = \frac{RT}{2F}\, kt. \qquad \text{......(5)}$$

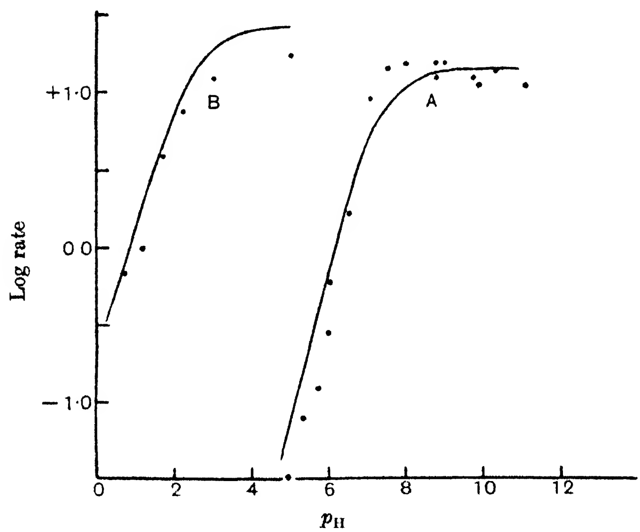


Fig. 2.

A. Isomerism of alloxan (monomol. velocity constant).
B. % autoxidation of dialuric acid per min.

The increase in negative potential should, therefore, be a linear function of the time. As has been already indicated, this agrees with observation. Equation (5) has been employed for the calculation of k from the electrode data at various $[H^+]$. In solutions of p_H greater than 7 the half-life period of alloxan at 30° appears to be only about one minute. The determination of k can, therefore, be only approximate. Nevertheless, as Table V and Fig. 2 indicate,

the velocity appears to be a function of the first acidic constant of alloxan. The curve in the figure represents the theoretical relation of log k to p_H were the velocity a function of the degree of dissociation of alloxan. Beyond p_H 10—coinciding with the intervention of the second (hypothetical) acidic constant—the potential drift falls away from a linear relation although the initial velocities remain of the same order of magnitude.

It may be added that the rates of change in p_H of mixtures of alloxan and sodium hydroxide give general confirmation of the velocities of change indicated above.

The autoxidation of dialuric acid.

To assist in defining the precautions necessary for the electrode studies, a few observations were made upon the rates of autoxidation of dialuric acid in buffered solutions. Between p_H 5 and 2 the rates were very great but fell away rapidly below this.

It was found that when the reaction was conducted by blowing oxygen through a solution of dialuric acid the velocity was greatly affected by the degree of agitation. Consequently, the following method was adopted. Solutions of 0·004 M dialuric acid (oxygen-free) were mixed in a closed chamber with five volumes of oxygenated buffer at zero time. Samples were removed at intervals, added to sufficient hydrochloric acid to depress autoxidation, and titrated in the absence of oxygen with 0·005 M iodine.

The rate of autoxidation was found to be roughly linear, with a tendency to fall away in the later stages. This was probably due to a significant diminution of the partial pressure of oxygen. The results were not closely reproducible, there being, apparently, uncontrolled catalytic effects. The latter did not however appear to be of the heavy metal type, since the addition of hydrocyanic acid was without notable effect. In Fig. 2 are plotted logarithms of the rates (percentage oxidation per minute) at various p_H values. The comparison curve gives the relation which would be expected were the velocity a direct function of the degree of dissociation of the dialuric acid.

Summary.

1. The equilibrium potentials of the reversible oxidation-reduction system dialuric acid-alloxan have been determined for the p_H range 1–6 and related to the equilibrium potentials of alloxantin observed by Biilmann and Lund.

2. The association constant of alloxantin has been derived from the electrode data.

3. Equilibrium potentials are not obtained in solutions of p_H above 6. This is probably due to the slow isomeric change of alloxan to alloxanic acid. Upon this assumption it has been possible to derive approximate equilibrium potentials for the range p_H 6–12.

4. The first acidic constants of dialuric acid and of alloxan have been determined. A second acidic constant of alloxan is inferred from the electrode behaviour of the system and a value for it derived.

5. Observations have been made on the relation to p_H of the rates of isomeric change of alloxan and of autoxidation of dialuric acid.

One of us (R. K. C.) is indebted to the Medical Research Council for a grant from which the expenses of this work have been defrayed.

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XII. NOTE ON THE DETERMINATION OF TRYPTOPHAN BY MEANS OF *p*-DIMETHYL-AMINOBENZALDEHYDE.

By WILLIAM JOHN BOYD.

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Tryptophan reacts with aldehydes in the presence of acids to give coloured solutions varying in colour according to the aldehyde used.

It is well known that in some cases the reaction requires the presence of an oxidising agent. Thus the colour reaction of formaldehyde with proteins in presence of sulphuric acid is promoted by the addition of a trace of ferric sulphate. Oxidising agents in general have a similar effect on the reaction of tryptophan with benzaldehyde and vanillin respectively, and it appears that this applies generally. Dakin [1906], however, states that the glyoxylic acid reaction (Adamkiewicz test) of tryptophan in presence of sulphuric acid does not require an oxidising agent, but this is true in measure of the reaction with other aldehydes. It can be shown that the presence of reducing substances such as hydrogen sulphide or formaldehyde retards the development of the glyoxylic acid coloration as well as that with other aldehydes.

In carrying out some work on the tryptophan content of fish-muscle protein by means of the method of May and Rose [1922] certain facts came to light which are of considerable importance in the application of this test since large errors may arise if they are ignored.

The May and Rose method is carried out as follows: 0·1 g. protein is added to a mixture of 50 cc. concentrated hydrochloric acid, 50 cc. water and 1 cc. of a 5 % solution of *p*-dimethylaminobenzaldehyde in 10 % sulphuric acid. The mixture is incubated at 36° for 24 hours and then allowed to stand for 24 hours or longer at room temperature. When tryptophan is present a blue coloured solution is obtained. The colour is compared by means of a colorimeter with the colour given by caseinogen under the same conditions, and the tryptophan content is calculated on the assumption that caseinogen contains 1·5 % of tryptophan. Jones, Gersdorff and Moeller [1924] have pointed out that different proteins are hydrolysed under these conditions at different rates and that a longer time must be given for development of the colour.

Holmes and Greenbank [1923] have studied the influence of temperature on the reaction of tryptophan, free and combined in proteins, with *p*-dimethylaminobenzaldehyde. They found that the development of the colour is much more rapid and complete at 37° than at room temperature and also that fading goes on concurrently with colour production. The colour is very stable at

room temperature but fades rapidly at 42°. They chose 37° as a suitable temperature and kept the mixture till a maximum intensity of coloration was obtained. This method gave in their hands good results for solutions of pure tryptophan but they do not state what standard was used. The conditions with proteins are very different since hydrolysis, colour development, and colour fading are all going on at once. For this reason a standard solution of pure tryptophan is not permissible in determining the tryptophan content of proteins if a high temperature is used.

Holmes and Greenbank have also found difficulty in the varying rate of hydrolysis of different proteins and favour the use of enzyme digests.

Some experiments were carried out with a view to explaining large variations in colour intensity obtained using equal volumes of the same trypsin digest of caseinogen or equal weights of the same sample of dry caseinogen. The relative intensities were sometimes as 7 : 10 and it was also found that the order might be reversed, that which at first was less intense becoming the stronger. These variations were traced to accidental differences in the illumination of the two tests. Also, sometimes delay occurred in the development of the colour in solutions of other proteins which caused it to be assumed that only a trace of tryptophan was present, an assumption subsequently disproved by development of considerable coloration. This was traced to the presence of small quantities of reducing substances. It was shown that development of the colour in solutions of pure tryptophan is retarded by (1) the presence of hydrogen sulphide or formaldehyde, (2) a layer of toluene over the reacting mixture, (3) absence of light. It was also found that addition of traces of an oxidising agent brings about rapid development of the colour at room temperature, and that exposure to bright light has a similar effect. The presence of pure isinglass does not hinder the development of colour, it is neither hindered by bubbling carbon dioxide through the reacting mixture in daylight, nor hastened by bubbling oxygen through the reacting mixture.

The following factors will now be considered in turn: (1) effect of reducing substances; (2) effect of oxidising agents; (3) effect of light.

Effect of reducing substances.

Herter [1905] has shown that the colour reaction of urine with *p*-dimethylaminobenzaldehyde (due to indole derivatives) is prevented by the presence of formaldehyde. Acree [1906] and Dakin [1906] also mention that excess of formaldehyde prevents the development of the coloration given by milk with formaldehyde in presence of sulphuric acid. In the course of work on cod-muscle protein it was found that, if this purified protein is soaked in a dilute solution of formaldehyde and then thoroughly washed with cold water, the Adamkiewicz reaction with glyoxylic acid in presence of sulphuric acid and the *p*-dimethylaminobenzaldehyde reaction in presence of hydrochloric acid become faint. The material appears to recover the power of giving positive tests with these reagents on prolonged storage.

These observations, together with the experiment with hydrogen sulphide already mentioned, show that the presence of reducing substances is a disturbing factor in the determination of tryptophan by the method of May and Rose and similar methods using other aldehydes. It follows too that excess of the aldehyde reagent will hinder the development of the colour and may cause colorimetric readings at a given time to be not truly proportional to the tryptophan present. In this connection it may be noted that Holmes and Greenbank found little difference in the results obtained, using 1 mol. of reagent for every mol. of tryptophan and using 10 mols. of reagent for every mol. of tryptophan, but they obtained the best results with 2 mols. It is evident that if only 1 mol. is present the aldehyde reagent will be gradually oxidised so that by the time all the tryptophan has been liberated there will not be the equivalent quantity of aldehyde to combine with it. On the other hand, too great an excess of reagent may retard the oxidative development of colour. Probably at the higher temperature, 37°, this is not so evident as at lower temperatures.

Effect of oxidising agents.

The addition of an oxidising agent at the same time as the reagent is added will obviously alter the aldehyde rapidly before it has time to combine with the tryptophan, especially as the latter is only slowly liberated from the protein. This is confirmed in practice when quantities of the order employed by May and Rose are used. The oxidising agent must be added after hydrolysis is completed.

In making qualitative tests in small volumes of liquid the coloration can be produced at once on addition of an oxidising agent. Small pieces of tissue can be quickly stained for tryptophan in this way. In these instances, however, probably only a small fraction of the tryptophan present is transformed.

In order to compare the coloration obtained with and without addition of an oxidising agent, tests were carried out by the method of May and Rose, using 0·1 g. of caseinogen and cod-muscle protein respectively. Duplicate tests were carried out similarly but 3 drops of 0·5 % sodium nitrite solution were added after 3 days and again after 3 days more. The colorimetric readings were made on the following day using a Duboscq colorimeter and a blue glass screen of the correct tint as standard. They are given in Table I.

Table I. *Comparison of the intensity of coloration obtained with and without addition of sodium nitrite solution.*

Substance	Time at room temp. after 1 day at 38°	Without addition of sodium nitrite	Sodium nitrite added
Caseinogen	7 days	22·9 mm.	15·0 mm.
	31 days	17·4 mm.	
Cod-muscle protein	7 days	34·8 mm.	17·6 mm.

The quantity of tryptophan present is in inverse proportion to the colorimetric reading.

From these results it is evident that the development of colour, without addition of sodium nitrite, is so slow that it does not in 31 days attain to the intensity obtained in 8 days on addition of sodium nitrite. Also it appears that if caseinogen is taken as a standard in order to determine the tryptophan content of cod-muscle protein, a higher result is obtained with sodium nitrite than without it.

If caseinogen is assumed to have 2·2 % tryptophan [cf. Jones, Gersdorff and Moeller, 1924] the values for cod-muscle obtained by the two methods are 1·45 % and 1·87 %. As already explained, variations in the intensity of the illumination in the one case may give variable results.

Similarly for edestin a tryptophan content of 3·50 % was found, which is considerably higher than the value obtained by May and Rose [1922] and by Jones, Gersdorff and Moeller [1924] without addition of an oxidising agent, the same tryptophan content of the caseinogen being assumed in each case.

Addition of a trace of nitric acid or of hydrogen peroxide has a similar effect to that of sodium nitrite.

Effect of light.

It has been mentioned already that the coloration produced in the May and Rose test varies according to the degree of illumination of the solution.

The following experiment illustrates the effect of light in promoting the reaction of tryptophan with *p*-dimethylaminobenzaldehyde. 0·1 g. edestin was added to a mixture of 100 cc. 18 % hydrochloric acid with 1 cc. of a 5 % solution of *p*-dimethylaminobenzaldehyde in 10 % sulphuric acid. This was allowed to stand at room temperature (10°) in the dark for 24 hours. A little gelatinous edestin remained undissolved and was removed by means of a glass rod. Equal portions of the clear colourless solution were decanted into three beakers of 5 cm. diameter. One of these was returned to the dark room, one was placed just inside a closed window and the third outside on the sill. The sky was cloudy but fairly bright. Within 1 minute the solution outside was distinctly blue, whilst no trace of blue colour could be detected in the other portions. In 30 minutes the solution outside was deep blue, that inside the window was faintly blue, whereas that in the dark was quite colourless. A drop of hydrogen peroxide rapidly developed colour in the last-mentioned solution. It is probable that the ultra-violet rays are the active agents since the interposition of glass or toluene makes a considerable difference in the rate of colour development.

From these considerations it appears that the best way to avoid the disturbing effects of varying illumination, and of reducing substances, is to add a little sodium nitrite, nitric acid or hydrogen peroxide to the test after hydrolysis is completed. For example 3 drops of 0·5 % sodium nitrite may be added to the test after incubation for 24 hours at 36° and 3 days at room temperature, and again after 3 days more, the colorimetric comparison being made next day.

The addition of sodium nitrite has been adopted by Fürth and Lieben [1920], using formaldehyde instead of *p*-dimethylaminobenzaldehyde. This addition is now shown to be advisable with the latter reagent.

Undoubtedly the ideal procedure would be to carry out the test on an enzyme-digest of the protein if the tints obtained with these digests were not so variable for different proteins. From some tests made on these lines it appears likely that the tryptophan content of cod-muscle is higher than the value given on page 81, and higher than that of caseinogen, but owing to the difficulty mentioned the results are uncertain.

Summary.

Errors can arise in the determination of tryptophan in proteins by the method of May and Rose (1) through unequal illumination of the reacting mixtures, and (2) through the presence of reducing substances such as hydrogen sulphide or aldehydes.

The development of the colour is an oxidation process which goes on slowly in dull light and more rapidly in bright light. It is not nearly complete in a period of 4 weeks in ordinary diffuse daylight in the laboratory. It can conveniently be hastened by adding a trace of an oxygen carrier or oxidising agent after hydrolysis of the protein.

In carrying out the test 3 drops of 0·5 % sodium nitrite solution should be added to the reaction mixture after 24 hours' incubation at 36° and 3 days at room temperature and again after a further 3 days, the colorimetric comparison being made next day or later.

By this modified method higher values for the tryptophan content of cod-muscle protein and edestin are obtained than by the unmodified method of May and Rose.

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XIII. THE ACTION OF INSULIN IN NORMAL YOUNG RABBITS.

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The early publications by the discoverers of insulin gave evidence that under the influence of insulin the glycogen content of the liver increased. Later, however, contradictory results were obtained. Dudley and Marrian [1923] state that, using normal mice, they found that the sugar disappearing from the blood under the action of insulin is not stored in the liver or the muscles as glycogen. Further they found that a dose of insulin big enough to produce convulsions depletes the liver and muscles of glycogen. Collazo, Händel and Rubino [1924], however, investigating the glycogen of liver and muscles in normal guinea-pigs 4 hours after the injection of 7 units of insulin, found a marked increase in both situations. Their animals were starved for 18 hours and received 3 g. of glucose by stomach tube before the injection of the insulin. The degree of rise in glycogen in these experiments both in liver and muscle was about 50 to 70 % above the controls. Cori [1925] found that starving rabbits showed no appreciable change in their liver-glycogen in the first hour of insulin action and that this was true whether the initial glycogen content was high or low and whether the fall in blood-sugar was large or small. In the second hour after insulin the liver-glycogen was found to remain constant or decrease. Similar results were obtained with mice, the average liver-glycogen of 16 injected animals being 39 % lower than the average of 16 controls. On the other hand completely phloridzinised and starving cats, dogs and rabbits were found to deposit liver-glycogen under the influence of insulin. Cori [1924] found an increased glycogen formation as a result of insulin action. Grevenstuk and Laqueur [1925], however, questioned this result. Frank and Hartmann [1926] criticised the findings of Grevenstuk and Laqueur, and Frank, Nothmann and Hartmann [1925] claim to have found an increase in liver-glycogen in fasting rabbits using small doses of insulin. Barbour *et al.* [1927], using a large number of standard rats, examined the changes in blood-sugar and muscle- and liver-glycogen induced by insulin. The effect produced depended on the dose of insulin. Thus, with 1 or 2 units per kg. body weight the liver-glycogen fell during the first 1½ hours and rose slightly after 2 hours: with 3 units per kg. there occurred a steadily progressive fall in the liver-glycogen. The muscle-glycogen of starving animals fell in all cases after insulin. In fed animals,

however, the total fall in liver-glycogen was almost balanced by a rise in the muscles. Cori and Cori [1928], after studying sugar utilisation in normal fasting rats, concluded that, "after insulin, the utilisation of absorbed sugar in the peripheral tissues (mainly muscles) is increased to such an extent that there is almost no sugar available for glycogen deposition in the liver." On this view, therefore, the main action of insulin is to bring about increased oxidation of glycogen in the peripheral tissues. Other experimenters found definite decreases in muscle-glycogen after insulin, for example, Dudley and Marrian [1923], Andova and Wagner [1924], and Kay and Robison [1924]. According to Hetenyi [1925] the "total sugar" of muscles falls after insulin. The work of Dale and co-workers [1926] has demonstrated that deposition of glycogen can be brought about in the eviscerated spinal cat by means of insulin, whereas without insulin perfusion with glucose does not lead to glycogen formation. Best, Hoet and Marks [1926] regard the immediate action of insulin as being a production of a more rapid combustion of sugar with a coincident further synthesis of glycogen.

It is apparent that as far, at any rate, as the liver-glycogen is concerned no final decision has been reached. The interpretation of any given series of results will naturally depend on the view taken of the glycogenic function. The two main views of the glycogenic function are the so-called storage theory and the secretory theory. The distinction between these views is fundamental: the former states that glycogen is deposited in the liver as a passive store which represents the excess of absorbed over utilised carbohydrate, and that the function of the liver is to keep the blood-sugar below certain levels. The latter regards glycogen as a true internal secretion and considers that the liver is constantly manufacturing this substance and liberating it as glucose in order to keep the blood-sugar above hypoglycaemic levels. The evidence for the secretory view is accumulating rapidly and has been fully discussed by Cramer [1928] and experimentally supported by Markowitz [1925].

A further matter which must be constantly kept in mind is the irreversibility of muscle-glycogen. Once glycogen is formed in the muscles it can no longer contribute to the upkeep of the blood-sugar. The experiments of Mann and Magath [1925] show that in hepatectomised dogs adrenaline can no longer bring about a rise in blood-sugar, in spite of the presence of glycogen in the muscles. From the point of view of available carbohydrate which could conceivably be of value in the relief of insulin convulsions it seems clear that muscle-glycogen is valueless. Strangely enough Chaikoff [1925] suggests that the condition for the recovery from convulsions is the presence of muscle-glycogen.

Dudley and Marrian [1923] concluded that muscle-glycogen was almost completely absent when convulsions occurred, but Chaikoff [1925] showed that this was not the case, a finding with which we entirely agree.

Experimental.

The first point which had to be considered was the choice of animals which could provide reliable controls. Barbour *et al.* [1927] state that rabbits are unsuitable because of the marked irregularity in the glycogen content of the tissues, even when every precaution is taken to have the animals fed and otherwise treated alike. This is certainly true of adult rabbits but it is not for very young ones, particularly if they are from the same litter. The values for muscle-glycogen are not as uniform as those for liver-glycogen. It was therefore decided to breed our own rabbits and carry out comparative experiments on members of the same litter. The animals were allowed to grow for periods of about 6 weeks, their diets being bran, oats and abundant green stuff as soon as they became independent of the mother. Occasionally there is some difficulty in keeping them healthy, as they are very susceptible to infections. Our best litters were obtained from primiparae, particularly if the young were all dominants.

The young animals were placed in a cage with no food for 24 hours before experiment. They were then divided into two groups, one being taken for controls. Only the most definitely healthy animals were taken.

Blood-sugar was determined by MacLean's method, heart-blood being generally taken.

Glycogen was determined in liver and muscle in the following manner. The animal was killed by a sharp blow on the back of the head, extended and the thorax and abdomen were rapidly opened. 0·2 cc. blood was taken immediately from the heart and transferred to the sodium sulphate used in the blood-sugar method. The thorax was packed with swabs and the liver quickly removed, the gall bladder detached and the liver weighed. Boiling 60 % potassium hydroxide (1 cc. to 1 g. tissue) was added. This process, from the time of killing the animal until the addition of the potassium hydroxide, did not occupy more than 90 seconds. In general the whole liver was worked up, but in a few cases part of it was taken for the estimation of fat. The liver was never minced but cut into small pieces with sharp scissors.

Meanwhile the posterior extremities were skinned and the gastrocnemii muscles removed, weighed and worked up for glycogen in the usual way. The separation of the muscles is easy in young rabbits because there is very little fat and one obtains what is almost all muscle tissue. The precipitate of glycogen was allowed to settle during 24 hours, filtered, washed twice with 66 % alcohol, twice with absolute alcohol and with absolute ether. The glycogen was dissolved in boiling water which was allowed to filter through into a volumetric flask. An aliquot part was hydrolysed in 1·8 % HCl, until no more glucose was produced. The period was usually 2 hours when the total glucose was not greater than 60 mg. After cooling and neutralising with KOH, the solution was made up to a known volume and filtered through a starch-free filter-paper.

The glucose was estimated by the method of von Issekutz and von Both

[1927] which depends on the reduction of potassium ferricyanide as in the Hagedorn and Jensen blood-sugar method, and the quantities have been so adjusted that amounts of glucose only up to 15 mg. can be estimated. The technique is particularly suitable for accurate glycogen estimations. In general, in this work, the quantities were adjusted so that no estimation involved more than 10 mg. of glucose. Many duplicates were carried out, always with the most satisfactory results.

The insulin used in these experiments was that prepared by Messrs Burroughs and Wellcome. Dilutions were made up in distilled water so that 1 cc. contained 1 clinical unit, and were used only on the day of preparation.

Exp. 1. Six young rabbits, 10 weeks old, from the same litter. Starvation for 24 hours before experiment. Three were used as controls and three were injected subcutaneously with 0·5 clinical unit of insulin. The first of the insulinised animals was killed 1 hour after the injection and the remaining two at the first sign of convulsions. The onset of convulsions in these young animals is always introduced by a period of incoordination followed by flaccidity. These are followed generally by a certain period of recovery, then a typical convulsion. In this experiment the animals were not allowed to go into convulsions but killed at the onset of flaccidity.

Blood for sugar estimation was taken from the heart. The fat content of the liver was also determined in this experiment.

Controls.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Liver-fatty acid (%)	Time after insulin (hrs.)
1	338	0 077	0·33	0·12	31	3·91	—
2	397	0·162	0·32	0·16	38	8·59	—
3	373	0·120	0·31	0 21	30	7·75	—
Insulinised animals (0·5 *unit in each case*).							
4	340	0·062	0·79	0·17	72	3·80	1
5	415	"0"	1·63	0·25	204	9·60	2½
6	414	"0"	1·76	0·18	248	6·68	3

It will be seen that the blood-sugars of Nos. 5 and 6 are given as "0."

This symbol indicates that the thiosulphate equivalent of the blood-sugar by MacLean's method was less than 0·2 cc. It was frequently noticed in these experiments that a blood-sugar of these vanishing proportions was not necessarily immediately associated with convulsions, and in the above animals there was merely loss of coordination and flaccidity.

In the insulinised animals the liver-glycogen displayed a rise, compared with that of the normal animals, of over 100 % in 1 hour and over 700 % in 3 hours. The muscle-glycogen did not show any significant variations. The values for the liver-fat gave no information at all. The iodine values were 83, 92 and 101 respectively for the controls, and 105, 90 and 118 for the insulinised animals, thus showing no definite change in the degree of saturation of the fatty acids.

Exp. 2. In this experiment the dose of insulin administered was 0·2 unit per animal. Four young rabbits from the same litter, 6 weeks old, were taken. Starvation for 24 hours before experiment.

Controls.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Remarks
1	565	0·090	0·60	0·06	124	—
2	427	0·110	0·15	0·19	21	—
			Insulinised animals (0·2 unit per animal).			
3	523	0·065	1·40	0·08	272	1 hr. 25 mins. after insulin
4	473	"0"	0·95	0·23	160	2 hrs. after insulin

In this experiment the same effect is seen as far as the liver-glycogen is concerned. The liver-glycogen in No. 1 was unusually high, but in spite of this there could be no doubt as to the very large increase. The results with the muscles were difficult to interpret.

In the next experiment the glycogen deposit at the beginning of convulsions was compared with that in animals after death following convulsions.

Exp. 3. Three young rabbits from the same litter, 6 weeks old. The dose of insulin was 0·5 unit per animal.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Remarks
1	384	0·11	0·27	0·43	41	Control
2	479	"0"	0·92	0·20	160	Slight convulsion 1¼ hrs. after insulin. Killed 1 hr. 40 mins. after insulin
3	427	"0"	2·03	0·15	292	Violent convulsions 1¾ hrs. after insulin. Died in convulsions 3 hrs. after injection

Again the very large rise in glycogen in the liver is seen, the animal actually dying in convulsions having about seven times as much in its liver as the control. This quantity of glycogen converted into glucose and discharged into the blood-stream would have been ample to avert convulsions and death for a considerable time. Whatever theory be accepted as to the action of insulin, it is quite clear that the glycogen is "locked" in the liver and not even the violent convulsions, with, probably, the stimulation of the adrenals, could in these animals bring about an effective glycogenolysis. The values for muscle-glycogen showed that, at any rate, death was not due to the absence of glycogen, as was suggested by Chaikoff [1925]. There was, however, definitely less glycogen per cent. in the insulinised animals than in the controls. Attention must be drawn to the fact that there was nearly twice as much glycogen in the liver of the animal allowed to die of convulsions as in that of the one killed at the onset of the hypoglycaemic attack. This, we consider, is clear proof that the action of insulin, in so far as it influences the deposition of glycogen, is independent of the convulsions. In fact we formed the impression that the

animal, as far as its glycogenic mechanism is concerned, was divided into two separate systems: one amassing glycogen in the liver and preventing its escape as glucose, and the other depleting such glycogen as was already present in the peripheral tissues. In these young animals the convulsions are never so violent as they are in adult animals and so the fall in muscle-glycogen is not so marked.

In the next experiment it was decided to find how far the very marked effect of insulin on the liver-glycogen could be influenced by the injection of adrenaline. It is well known that adrenaline exerts an antagonistic action to insulin by stimulating glycogenolysis and so preventing hypoglycaemia. The old observation of Gatin-Gruzewska [1906] that adrenaline depletes the glycogen of the body is well known, but a considerable time of action and repeated injections are necessary to produce this effect. Pollak [1909] found that repeated injections of adrenaline into glycogen-free rabbits brought about a deposition of glycogen of the same order as that found in carbohydrate-fed animals.

Drummond and Paton [1904] obtained similar results. Kuriyama [1918] found an increase of glycogen in the livers of fasting rabbits after injections of adrenaline, and Cori [1928] found a marked increase in rats. Muscle-glycogen is found to fall and it has been suggested that adrenaline brings about an increased breakdown of muscle-glycogen to lactic acid, which then can be built up again to glycogen in the liver. In a recent communication on the action of adrenaline in depancreatised dogs Chaikoff and Weber [1928] claim to have shown that it can bring about a marked gluconeogenesis from fat. There is no doubt that adrenaline can produce a very marked hyperglycaemia in animals that have been starved for long periods or otherwise treated until the liver contains only very small amounts of glycogen. If, as seems very probable, these experiments indicate that adrenaline stimulates gluconeogenesis and at the same time glycogenolysis, then its effect on insulin action will depend upon the difference between these two processes.

What we wished to determine was whether any evidence could be obtained that adrenaline influenced the action of insulin directly.

Exp. 4. Four young rabbits from the same litter, 6 weeks old. Starvation 24 hours.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Remarks
1	384	0·110	0·27	0·43	41	Control
2	363	0·256	0·38	0·28	49	0·25 mg. adrenaline subcutaneously. Killed 1¾ hrs. later
3	366	"0"	1·56	0·36	190	0·5 unit insulin subcutaneously. Killed at onset of convulsions 1 hr. 35 mins. later
4	364	0·146	1·34	0·20	182	0·25 mg. adrenaline, 0·5 unit insulin at same time. Killed 2 hrs. later

Exp. 5. Two rabbits from the same litter, 6 weeks old. Starved 24 hours.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Remarks
1	328	0·040	0·34	—	38	Control: blood-sugar unusually low
2	402	0·062	3·86	—	465	2.12 p.m. 0·5 unit 5.38 p.m. 0·5 unit 6.45 p.m. Convulsion 6.55 p.m. Convulsion. 1 mg. adrenaline Killed 2 hrs. later, after complete absence of convulsions for 2 hrs.

It is clear from these experiments that 0·25 mg. adrenaline acting for 1¾ hours had no effect on the total liver-glycogen of animal No. 2, experiment 4, although there was hyperglycaemia: but there was a suggestively lower muscle-glycogen. No. 3 showed the usual large increase in liver-glycogen. Animal No. 4 showed that 0·25 mg. adrenaline could not prevent the accumulation of glycogen under the action of insulin, although sufficient sugar was liberated to prevent all hypoglycaemic manifestations. When we consider that, throughout the period of 2 hours after the injections in animal 4, there was a high discharge of sugar from the liver and probably an increased peripheral utilisation, and that in spite of this the accumulation of glycogen was of the same order as in animal 3, it seems not unreasonable to suppose that the adrenaline had contributed to the process of glycogenesis as well as given rise to an increased glycogenolysis.

In experiment 5, 1 mg. of adrenaline, whilst bringing about complete recovery from the insulin convulsions, did not do so by inhibiting the effect of insulin on the liver-glycogen. In fact the increase in liver-glycogen was to about 12 times the control value, in spite of a continuous discharge of sugar for 2 hours.

Up to this point, then, our results indicate that, in the starving young rabbit, insulin brings about a large increase in liver-glycogen and possibly a decrease in muscle-glycogen: further, that the increase in the liver can proceed in spite of the fact that the animal may be approaching death, which could be averted if this glycogen were liberated. It has also been seen that in these young animals death may occur in insulin convulsions from exhaustion, without the glycogen of the muscles having been used up. In addition it has been found that adrenaline in doses sufficient to produce a powerful hyperglycaemia does not necessarily deplete the glycogen in the liver, nor does it inhibit the glycogen increase following the injection of insulin.

Our next series of experiments was carried out with the object of finding what agents could bring about an inhibition of the glycogenesis induced by insulin. From several previous experiments we had satisfied ourselves that anaesthesia with ether or chloroform produces in rabbits a very high and lasting

hyperglycaemia, which involves the whole cardio-vascular system. The question as to whether this effect is due to a stimulus to glycogenolysis or to an inhibition of insulin action presented itself.

It was decided to use ether because of the less severe strain on the vascular system, chloroform being definitely dangerous to such young animals as we were using.

In examining the results obtained it must be understood that in no case in which we have injected insulin into young rabbits has there been any question of the immense rise in liver-glycogen.

Exp. 6. The animals used were 6 weeks old and from the same litter as those in experiment 5. The two rabbits of 5 are included in the following table for comparison. The plan of experiment was to anaesthetise an animal when it developed insulin convulsions and to find the effect of insulin in another animal when it was fully under ether. Muscle-glycogen was not determined.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Liver-glycogen %	Liver-glycogen mg.	Remarks
1	328	0·040	0·34	38	Control
2	402	0·062	3·86	465	Insulin and adrenaline, *vide* Exp. 5
3	342	"0"	0·69	84	2.13 p.m. 0·5 unit insulin 4.30 p.m. Convulsion Open ether—complete recovery 5.42 p.m. 0·5 unit insulin 6.45 p.m. Convulsion Open ether—complete recovery 7.17 p.m. Convulsion Open ether—complete recovery 7.40 p.m. Killed
4	518	0·104	0·49	84	11.30 a.m. Blood-sugar 0·131 g. per 100 cc. 11.45 a.m. Open ether and kept under 11.46 a.m. 0·5 unit insulin 11.55 a.m. Blood-sugar 0·122 g. per 100 cc. 12.45 p.m. Blood-sugar 0·117 g. per 100 cc. 1.30 p.m. 0·5 unit insulin 1.35 p.m. Blood-sugar 0·077 g. per 100 cc. 3.15 p.m. Killed after 3½ hrs. anaesthesia

The first point which emerges from this experiment is that the insulin convulsions were completely stopped by the ether anaesthesia. In the case of animal 4 the onset of convulsions was entirely prevented. With animal 3 the anaesthesia was light, so that convulsions occurred when it wore off somewhat: but in the case of 4 the continuous and deep anaesthesia almost entirely inhibited the action of the insulin, thus, in 3½ hours after the two injections of 0·5 unit each, the blood-sugar had only fallen from 0·131 to 0·104 g. per 100 cc. Further, in neither case did there occur the very large rise in liver-glycogen which occurs invariably after insulin alone. There was, it is true, over twice as much glycogen in the livers of 3 and 4 as in the control, but this is a very small effect compared to that, for example, in No. 2.

We conceive that in animal 3 the glycogen content of the liver was rising and would have continued to do so had the animal been allowed to go on living

for as long as it could under the circumstances. The action of the insulin would come in as the effect of the ether wore off.

In animal 4, on the other hand, the continuous anaesthesia tends to promote a continuous flow of sugar from the liver whilst the insulin ineffectually antagonises this by exerting a "locking" action on the liver. This effect is different from that of adrenaline, for, as we have seen in experiments 4 and 5, an injection of this substance sufficient to prevent convulsions produces hyperglycaemia and does not prevent the insulin from promoting a large deposition of glycogen in the liver. It seems to us that the effect of the anaesthetic is due to a toxic action on the liver cells, making it difficult for them to elaborate glycogen from exogenous or endogenous carbohydrate.

In animal 3 there must have been at 4.30 p.m. a large increase in liver-glycogen over the value at the outset, so that the anaesthesia must have liberated it again, this happening each time the convulsed rabbit was given ether. In animal 4 there did not occur any considerable hyper- or hypo-glycaemia throughout the 3½ hours of the experiment, hence the inability to lay down any largely increased amount of glycogen must have been due to some effect on the liver itself.

The very powerful liberation of sugar which takes place in rabbits under ether is shown in the following experiment on an adult animal.

Time after ether anaesthesia (mins.)	Blood-sugar, g. per 100 cc. 0	10	40	48	58
Peripheral vein	0·129	0·193	0·327	0·383	0·383
Inferior Vena Cava	—	—	0·330	0·340	0·378
Portal vein	—	—	0·293	0·327	0·362

The fact that this effect was absent in animal 4, experiment 6, shows that glycogenolysis was at its normal level and hence the failure to lay down any greatly increased quantity of glycogen localises the inhibition in the liver itself.

The failure of insulin to produce its typical effects in certain cases of very severe diabetes may be analogous to this effect of ether. The intense acidosis and poisoning with the toxic products of abnormal metabolism may produce this antagonism to a successful action of insulin by rendering the cell incapable of forming glycogen. Thus Sevringhaus [1926] found that injections of sodium acetoacetate into fasting rabbits retarded the blood-sugar-lowering action of insulin. Koehler [1926] found that in diabetics with well-marked acidosis (blood-p_H 6·8 to 7·2) doses of 100 to 300 units in 24 hours had no marked effect. The partial loss of tolerance to carbohydrate, induced by the ingestion of NH_4Cl, $CaCl_2$ or $NaHCO_3$ in big doses, is probably due to a similar toxic condition of the hepatic cell. The same is probably true of the intolerance to carbohydrate arising in starvation.

If this view of the action of ether is correct, its effect in preventing insulin convulsions and the accumulation of glycogen in the liver becomes understandable.

Adrenaline acts by liberating hepatic carbohydrate, at the same time probably increasing gluconeogenesis, and so does not, within the period of our experiments, interfere with the action of insulin in promoting an accumulation of liver-glycogen, although the blood-sugar may be maintained at high levels. Ether, on the other hand, prevents insulin from exerting its typical effect, by making it difficult or impossible for the liver cells to transform sugar to glycogen.

The next matter which was examined was the question as to how far the action of insulin could be influenced by convulsions. There seems to be no doubt that convulsions in a muscle deplete its glycogen, although there is some evidence that a muscle can respond to induction shocks even when its glycogen content has been reduced to vanishing proportions by injection of convulsants [Olmsted and Harvey, 1927]. Now in our young animals the convulsions induced by insulin are not powerful enough to deplete either muscle- or liver-glycogen. What we wished to determine was whether convulsions produced by, say, strychnine sulphate could in the presence of insulin prevent the accumulation of glycogen in the liver. Two types of experiment were possible: (1) to induce strychnine convulsions during the action of insulin, and (2) to inject insulin during and between strychnine convulsions.

Exp. 7. Seven young rabbits from the same litter, 6 weeks old. Starvation 24 hours before experiment in each case.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Remarks
1	451	0·113	0·59	0·30	78	Control
2	448	0·090	0·32	0·14	56	2.48 p.m. 0·2 mg. strychnine 2.52 p.m. Convulsion—typical 3.20 p.m. Died after 28 mins of convulsions
3	457	0·109	0·26	0·19	50	1.35 p.m. 0·05 mg. strychnine 2.06 p.m. 0·10 mg. strychnine 2.36 p.m. 0·10 mg. strychnine 2.54 p.m. 0·20 mg. strychnine 2.57 p.m. Convulsion 3.34 p.m. Died after 37 mins. of convulsions
4	461	0·058	1·37	0·16	230	2.50 p.m. 0·5 unit insulin 5.02 p.m. 0·2 mg. strychnine 5.12 p.m. Strychnine convulsions 5.13 p.m. Died—11 mins. after injection of strychnine
5	442	0·113	1·90	0·15	374	2.00 p.m. 0·5 unit insulin 3.05 p.m. Hypoglycaemic reaction 3.21 p.m. Insulin convulsion with recovery 3.22 p.m. 0·2 mg. strychnine 3.27 p.m. Insulin convulsion with recovery 3.34 p.m. Strychnine convulsions —very violent 3.39 p.m. Died—17 mins. after injection of strychnine

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Muscle	Liver-glycogen (mg.)	Remarks
6	454	0·110	0·46	0·15	79	5·28 p.m. 0·2 mg. strychnine 5.50 p.m. Violent convulsions 5.51 p.m. 0·5 unit insulin 6.14 p.m. 0·2 mg. strychnine 6.30 p.m. Died—62 mins. after first dose of strychnine and 39 mins. after injection of insulin
7	453	0·056	0·26	0·21	46	6.59 p.m. 0·2 mg. strychnine 7.32 p.m. Convulsion with recovery 7.53 p.m. 0·15 mg. strychnine 8.04 p.m. Strychnine convulsion. 0·5 unit insulin 8.40 p.m. Strychnine convulsion ceased 8.56 p.m. 0·2 mg. strychnine 9.06 p.m. Intermittent convulsion 9.21 p.m. Killed—after 3 hrs. 22 mins. strychnine action and 1 hr. 17 mins. insulin action

Several facts emerge from this experiment. First, it is clear that these young animals can die after convulsions lasting over 30 minutes without losing all the glycogen from the liver or muscles, or, indeed, depleting it to any considerable extent. This is probably due to the fact, already mentioned, that the convulsions are not as violent as in an adult animal and that the young animals die of exhaustion. Nos. 2 and 3 illustrate this point. It is also to be observed that the period of convulsion was in both animals about half an hour.

In the cases of Nos. 4 and 5 the strychnine was injected when they were in a definitely hypoglycaemic condition and it was found that both animals died extremely rapidly. The effect was very striking, in No. 4 death followed in 1 minute after the onset of convulsions and in No. 5 in 5 minutes. In Nos. 4 and 5 the hypoglycaemia had been remedied by the strychnine convulsions, the values being 0·058 % and 0·113 % respectively. Increases were observed, moreover, in the glycogen in the liver of the same order as that when insulin acts alone, and the muscles contained amounts not significantly different from those of other young animals. Our attitude to the muscle figures is governed by the principle that we are in search of big effects and are loath to base hypotheses on small absolute changes. One thing, however, is clear, namely, that these animals did not die from hypoglycaemia or from depletion of muscle- and liver-glycogen. Of course in both cases the strychnine convulsions only lasted from 1 to 5 minutes, so that one could hardly expect much effect, but the fact that death followed so quickly seems to point to some hypersensitivity to the alkaloid in tha insulinised animal.

When, however, we examine the results obtained from animals 6 and 7 the picture is quite different in several respects. In animal 6, 0·2 mg. strychnine brought about violent convulsions in 22 minutes. 0·5 unit insulin was at once injected and the animal had no further convulsion during the succeeding 24 minutes. An additional 0·2 mg. strychnine was then injected and the

animal died in 16 minutes, that is, 62 minutes after the injection of the first dose of alkaloid and after the insulin had been acting for 40 minutes.

In animal 7, 0·2 mg. strychnine induced convulsions in 33 minutes, but recovery soon occurred. Another dose of 0·15 mg. induced violent convulsions in 11 minutes, when 0·5 unit insulin was injected. Convulsions of the strychnine type continued for 36 minutes and then ceased. After 16 minutes without any convulsion a further 0·2 mg. strychnine was injected and in 10 minutes violent intermittent convulsions set in. The animal was killed 15 minutes later, that is, 3 hours, 22 minutes after the first injection of strychnine and 1 hour, 17 minutes after the injection of insulin. In neither of these animals did a decided hypoglycaemia occur, the heart blood-sugars being 0·110 % and 0·056 %. As seen from animals 2 and 3, strychnine does not bring about a hyperglycaemia, so there is no analogy to the actions of adrenaline and ether, although the convulsions may conceivably stimulate the adrenals to some extent. Examining the results further, it is readily seen that the glycogen contents of both liver and muscle showed no significant departure from those of untreated animals, thus contrasting strikingly with the results in the cases of animals 4 and 5.

It appears then that during the violent convulsions following strychnine, insulin does not bring about its typical effect. Death, when it occurs in these conditions, does not result from the action of insulin but from that of the alkaloid.

It is tempting to imagine some interference arising from the action of the strychnine on the spinal cord, but much further evidence is necessary. There is, indeed, some evidence that insulin exerts its effect *via* the cord, but it is still only in the presumptive stage.

Discussion.

We take the provisional view that the action of insulin is to prevent the discharge of glycogen from the liver. We conceive that the process of absorption into the liver of glycogen-producing substances goes on from the gut and the blood as freely in the starving insulinised animal as in the control, but for some reason yet to be discovered the process of glycogenolysis is prevented. Peripheral utilisation continues and the blood-sugar reaches levels at which symptoms appear. This "locking" effect on liver-glycogen can be readily and strikingly demonstrated in young fed rabbits as well as starving animals.

Exp. 8. Two young rabbits from the same litter, 6 weeks old. Taken off food during the period of experiment only.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (g.)	Remarks
1	297	0·156	9·94	0·66	1·39	Control
2	373	"0"	9·26	0·31	2·04	1.05 p.m. 0·5 unit insulin 3.00 p.m. 0·5 unit insulin 5.25 p.m. 0·5 unit insulin 7.05 p.m. Convulsion with recovery 7.15 p.m. Killed

Exp. 9. Two young rabbits from the same litter, 6 weeks old. Taken off food during the period of experiment only.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Liver-glycogen (g.)	Remarks
1	542	0·143	1·56	Control
2	517	"0"	1·03	1.10 p.m. 0·5 unit insulin
				4.30 p.m. 0·5 unit insulin
				5.15 p.m. Convulsion
				5.30 p.m. Convulsion
				5.40 p.m. Convulsion
				5.43 p.m. Killed

Here we have animals, No. 2 in each experiment, which are *in extremis* for the want of blood-sugar and which possess between 1 and 2 g. of glycogen in the liver. Experiment 8 shows also the very much lower muscle-glycogen of the insulin-treated animal. The blood contained practically no copper-reducing substance and the liver was incapable of yielding any to it.

That in the fed animal it takes much longer and much larger doses of insulin to produce hypoglycaemia than in the starved animal we conceive as due to the difficulty of increasing the glycogen in a liver already highly charged with it. Thus the removal of blood-sugar would depend largely on peripheral utilisation. In the last two experiments there did not occur any significant change in the liver-glycogen, the sum of the two controls being 2·95 g. and in the insulinised animals 3·07 g.; so that if we accept the idea that insulin "blocks" the release of glycogen we must also be prepared to consider, in the fed animal, the probability of a factor relating to the regulation of absorption, because the gut in the fed animals was full of carbohydrate and other food in process of digestion. It seems reasonable to suppose that when the liver-glycogen reaches a certain level it is impossible to raise it further, so that the glycogenic function would become a self-limiting process. Insulin acting in these circumstances would block the outward delivery of sugar and the liver itself would inhibit any further uptake of carbohydrate from other sources. The probable existence of a limiting glycogen content in the liver, characteristic for each animal, can be demonstrated by an experiment using rabbits fed on a diet containing an excess of carbohydrate for varying periods. The following was such an experiment.

Exp. 10. Seven adult rabbits were used. Two were kept as controls on their usual diet of bran, oats and green vegetables. The remaining five were given carrots and greens for the periods indicated. They were then killed and the livers and muscles analysed for glycogen and fat. The fat was estimated to see if the excessive carbohydrate was perhaps stored as fat.

	Glycogen (%)		Fatty acid (%)		
No.	Liver	Muscle	Liver	Muscle	No. of days on carrots
1	2·60	0·40	—	1·65	Control
2	0·40	0·26	4·03	2·02	,,
3	6·30	0·41	2·75	1·49	2
4	11·29	0·05	2·07	1·29	7
5	7·54	0·21	2·74	1·31	12
6	4·90	0·11	2·62	1·68	15
7	8·60	0·06	2·68	1·20	31

We take these figures to show that on such a diet the liver-glycogen cannot be made to exceed about 11 %, and although we have not yet tried this in adult rabbits we think that the injection of insulin would not bring about any higher level than this. The view, then, put forward is that the absorption of sugar from the gut or the formation of sugar by gluconeogenesis is governed by the amount of glycogen already present in the liver. Insulin, by "locking" the liver-glycogen, tends to prevent both the processes of absorption and of gluconeogenesis. It is not, we suggest, the insulin as such which does this, but it is the result of the accumulation of glycogen. We conceive that it is the actual presence of the colloid glycogen which imposes a self-limiting action on the liver.

On this view the action of insulin might be explained as due to two factors: (*a*) the blockage of carbohydrate in the liver, and (*b*) the self-limiting mechanism of the liver.

Let us consider briefly the application of this view to the cases of (1) the starving animal, (2) the fed animal, and (3) the diabetic.

(1) The starving animal, with its small amount of glycogen in the liver, keeps up its blood-sugar by absorption from the gut and later by gluconeogenesis from protein and fat (the evidence for the latter source of carbohydrate is doubtful and will be the subject of a further communication). The endogenous internal secretions of the pancreas, thyroid and adrenal control this process so that an optimum balance is struck, conserving the body tissues as far as possible. Insulin given to such an animal blocks the release of carbohydrate from the liver, but offers no obstruction to absorption and gluconeogenesis, because the liver-glycogen is still far from the limiting value. The result is a large rise in liver-glycogen. And, as we have seen, this occurs even when adrenaline is acting strongly. The necessary and sufficient condition for this action of insulin is a healthy liver.

(2) In the animal which already possesses a high liver-glycogen the discharge of sugar into the blood is as steady as and not at a much higher level than in the starving animal. This discharge is obviously not accompanied by an equal replenishment, otherwise the liver-glycogen would not fall as we know it does. This, in our view, is due to a limitation of absorption and stoppage of gluconeogenesis. In such an animal insulin, although blocking the discharge from the liver, does not secondarily bring about much change in the liver-glycogen, because the high glycogen content has imposed the self-limiting process on the liver. Thus the animal goes into hypoglycaemia much more slowly, since the disappearance of peripheral sugar will depend mostly on peripheral utilisation. This continues until the animal may die in convulsions without liberation of sugar from the liver.

(3) In the diabetic subject absorption of carbohydrate goes on rapidly from the gut because of the low glycogen values in the liver and the loss of blockage power, due to the deficiency of insulin. Gluconeogenesis proceeds at a rate which is much greater than in the starving animal because of the loss

in conserving power. The R.Q. is low because of the high degree of gluconeogenesis counterbalancing the high R.Q. of carbohydrate utilisation. The height of the R.Q. under any circumstances must be the algebraic sum of these processes. Insulin, provided the liver cells are not damaged and are capable of elaborating glycogen, blocks the discharge of carbohydrate from the liver, the liver-glycogen increases until as a consequence excessive absorption is stopped and gluconeogenesis is inhibited. The R.Q. now rises because the latter process is controlled. The condition for effective action is again the condition of the liver cells.

The whole problem, therefore, resolves itself into how insulin "blocks" the liver-glycogen.

There does not appear to be any evidence that insulin increases metabolic rate more than can be brought about by normal effective metabolism. It has always seemed anomalous that the diabetic should have a more or less normal metabolic rate, and that even when his metabolic processes are controlled by means of insulin the total metabolism shows no significant increase. This, it seems to us, can only be explained by a readjustment of the partition of energy among fat, protein and carbohydrate, which comes about by prevention of uncontrolled release of carbohydrate from the liver and the consequent depression of gluconeogenesis.

Summary.

1. Young rabbits, 6 to 10 weeks old, of the same litter, are well adapted to comparative experiments with insulin. When starved for 24 hours they show a very satisfactory uniformity in their liver-glycogen. The average of twelve such animals from various litters was 50 mg. glycogen per liver.

2. Comparing the glycogen content of such rabbits, starved for 24 hours and treated with insulin, with that of controls from the same litter, there invariably occurred a very large rise in liver-glycogen. The average for twelve such animals was 237 mg.

3. Young rabbits treated with insulin may die in hypoglycaemic convulsions and still possess a very much increased liver-glycogen and a very considerable muscle-glycogen.

4. Adrenaline, whilst relieving hypoglycaemia, does not interfere with the essential action of insulin in increasing liver-glycogen.

5. Light ether anaesthesia relieves the convulsions due to hypoglycaemia. During continuous deep anaesthesia insulin cannot bring about the usual very large increase in liver-glycogen. This is considered to be due to a toxic action on the liver cell.

6. The hypoglycaemic animal is very susceptible to strychnine and dies in a very much shorter time than the normal animal. Continuous strychnine convulsions can prevent the typical action of insulin in promoting an increased glycogen content of the liver.

7. In the fed animal insulin does not bring about an increase in liver-glycogen and the animal goes into hypoglycaemic convulsions with large quantities of glycogen in the liver. Muscle-glycogen is definitely lowered.

8. These results are discussed and a theory of insulin action is suggested, *viz.* that the immediate effect of insulin is to inhibit the release of liver-glycogen. All other effects are regarded as secondary to this. The application of this view to certain cases is discussed.

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XIV. AN APPLICATION OF THE METHOD OF HAGEDORN AND JENSEN TO THE DETERMINATION OF LARGER QUANTITIES OF REDUCING SUGARS.

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The method of Hagedorn and Jensen [1923] has become widely used for the estimation of reducing sugar in blood. The method depends upon the fact that potassium ferricyanide is reduced to ferrocyanide when heated in alkaline solution with certain reducing substances. In the Hagedorn and Jensen procedure the ferrocyanide formed in this way is precipitated as the double potassium zinc salt, and the residual ferricyanide is estimated by adding an excess of potassium iodide and acidifying. The ferricyanide is reduced quantitatively by the iodide, and equivalent iodine is liberated according to the equation:

$$2H_3Fe(CN)_6 + 2HI = 2H_4Fe(CN)_6 + I_2.$$

The iodine is titrated with thiosulphate.

This method based on ferricyanide as the substance reduced has an inherent advantage over copper methods which will be referred to later. In the original form it was devised for estimating the reducing sugar in the filtrates from 0·1 cc. samples of blood. The capacity is accordingly very small, the upper limit being 0·385 mg. of glucose. Small volumes of solutions are used, and the essential reactants are present in low concentration so that the introduction of impurities may cause relatively large errors. For the purposes of my own work the range of the original method was inconveniently narrow, and the present modification makes possible the estimation of about ten times as much sugar. The new procedure has been standardised for maltose as well as for glucose. No essential change from the original method has been made, except as regards the concentrations and volumes of the various reagents employed.

The following solutions are used in the modified method. The chemicals used were of the "Analytical Reagent" quality, as supplied by the British Drug Houses, Ltd., unless otherwise stated.

Solution A.

Potassium ferricyanide	8·25 g.
Anhydrous sodium carbonate ...	10·6 g.

Make up to 1 litre with distilled water. This solution should be kept for 2 or 3 days before use and it must be stored in a bottle provided with an opaque jacket. In making the solutions for the standardisation data the potassium ferricyanide was twice recrystallised but as no significant difference is found when the untreated "A.R." product is used, this is not considered necessary.

Solution B.

Potassium iodide	...	...	12·5 g.
Zinc sulphate ...	...	...	25·0 g.
Sodium chloride ...	...	...	125·0 g.

Make up to 500 cc. with distilled water. Traces of iodine appear in this solution on storing. This iodine should be removed before using by filtering through two thicknesses of filter paper.

Solution C.

5 cc. glacial acetic acid, diluted to 100 cc. with distilled water.

Solution D.

Soluble starch solution, saturated with sodium chloride. 1 g. Merck's soluble starch in about 20 cc. cold water is washed into 60 cc. of boiling water. This is boiled for 2 minutes, 20 g. sodium chloride are added and the solution is allowed to cool. It is then made up to 100 cc. This solution keeps for several months.

Solution E.

An approximately $N/75$ sodium thiosulphate solution which is used in a 10 cc. burette, graduated into 0·02 cc. divisions. In practice it is found convenient to make up about 10 litres of this solution (containing 3·33 g. sodium thiosulphate per litre) using boiled-out water. It is then stored in a bottle, protected from atmospheric carbon dioxide by a soda-lime tube, and the solution is run through a siphon to the burette. When stored in this way the solution keeps well. At first it should be standardised each day but after the first week it need only be standardised at intervals of 3 or 4 days.

The thiosulphate solution is most conveniently standardised against a potassium iodate solution. This solution is made up by weighing out carefully a quantity of about 0·80 g. of desiccated potassium iodate and making it up to a volume of 1 litre in a volumetric flask standardised at the temperature obtaining. A 5 cc. pipette of slow delivery is standardised by weighing four or five deliveries of water at a known temperature. (The tip should, of course, be drained against the side of the collecting vessel for a standard time.) With this pipette 5 cc. of the standard iodate solution are run into a boiling-tube. To this are added 5 cc. of a 2 % solution of potassium iodide and then it is acidified by the addition of 3 cc. of 5 % acetic acid (solution C). The iodine liberated is titrated with the thiosulphate solution, adding a drop of the

starch solution D when the colour becomes a pale yellow, and titrating to the disappearance of the blue colour. The volume of thiosulphate required when the normality is 0·01333 ($N/75$) is about 9 cc. The value for the normality is found from the expression

$$\frac{\text{g. K iodate per litre} \times \text{vol. of pipette (cc.)}}{35{\cdot}67 \times \text{vol. of thiosulphate required (cc.)}}.$$

The results from this method were found to give exact agreement with the method described by Cole [1928] in which the thiosulphate is standardised against a standard acid.

Procedure for determination of sugar.

The estimations are carried out in boiling-tubes, 1 × 7 in. Glass bulbs with about an inch of tubing left attached are used as covers for the boiling-tubes.

5 cc. of solution A (alkaline ferricyanide) are pipetted into a boiling-tube, allowing the pipette to drain a standard time. To this are added 5 cc. of the solution whose reducing power is to be determined. (Note: if less than 5 cc. is added, sufficient water is added to make up the difference in volume.) A water blank is now made up by pipetting 5 cc. of solution A into a second tube and adding 5 cc. of distilled water. It is important that any drops of liquid adhering to the walls of the tubes should be mixed in, and this is best done by inclining the tube and slowly rotating it.

The tubes are now covered with glass bulbs and placed for 15 minutes in a boiling water-bath 2 or 3 in. deep. After this they are cooled for 3 minutes in cold running water.

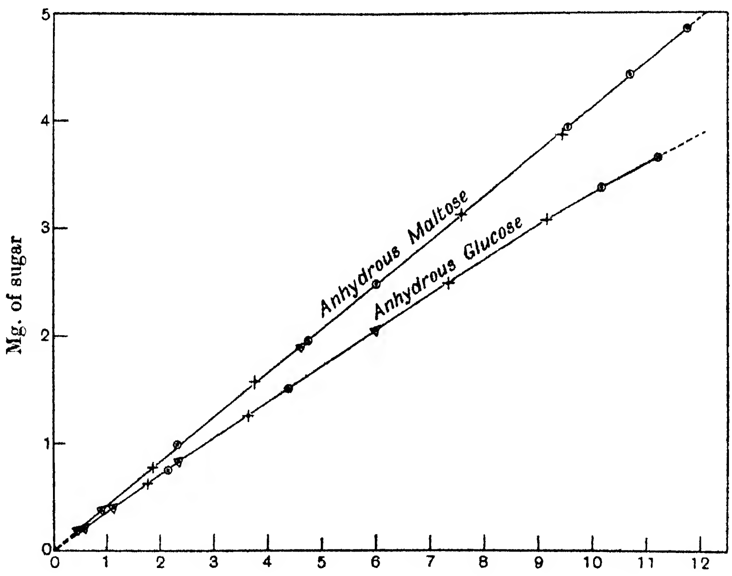
The remaining operations are concerned with the estimation of the residual ferricyanide. 5 cc. of solution B are added. This is conveniently run in from a rapid pipette as the volume need not be precise. A white flocculent precipitate appears and at the same time iodine is set free. 3 cc. of solution C are now added (also from a rapid pipette). The reduction of the ferricyanide is now completed and the iodine liberated is titrated against the standardised thiosulphate.

The titration is carried out in the same boiling-tube. Mixing during the titration is effected by shaking the tube with a circular motion. The titration should be carried out in a regular manner. Thiosulphate is run in slowly until the liquid has a pale yellow colour; 3 drops of the starch indicator are then added and thiosulphate is added drop by drop until the sudden disappearance of the blue colour[1]. The end-point is very sharp and it is advantageous to fit the burette with a fine tip which has been coated with paraffin wax as this makes the drops smaller and prevents the thiosulphate from wetting the tip.

[1] An "overshot" end-point may be regained by adding counted drops of iodine (about $N/75$ in KI) from a fine dropping pipette until the blue colour returns, titrating to the end-point with thiosulphate, and deducting from the reading the thiosulphate equivalent of the added iodine which is found by repeating the operation.

The difference between the water blank value (WB) and the reading obtained with the experimental solution (R) gives the thiosulphate equivalent of the ferricyanide reduced by the experimental solution. These reduction values have been determined for different amounts of added reducing sugars, maltose and glucose, and the results are shown in the standardisation graph, Fig. 1. For convenience in calculation the results are given in terms of $N/100$ thiosulphate, although approximately $N/75$ is used in the titration. Thus if the normality of the thiosulphate used for a particular estimation is 0·01351, then the $N/100$ thiosulphate equivalent of the ferricyanide reduced is $(WB - R) \times 0{\cdot}01351$ cc. The amount of sugar corresponding to this reduction is either read from the graph, or in the case of maltose it may be calculated from a factor.

In practice the water blank value need be determined only once or twice a day. Estimations can be done conveniently in batches of five or six and in this way it is possible to make 18 estimations an hour when facility in the technique has been acquired.



cc. $N/100$ thiosulphate equivalent to ferricyanide reduced (WB – R)

Fig. 1.

The heating period for reduction.

In order to follow the reduction after different periods of heating, five boiling-tubes were set up each containing 5 cc. of solution A and 5 cc. of a maltose solution containing 2·5 mg. These were placed in the boiling water-bath and allowed to remain for 5, 10, 15, 20, and 30 minutes respectively. On removal each tube was cooled for 3 minutes and the residual ferricyanide was estimated in the usual way. The results are given in Fig. 2 in which the amounts of ferricyanide reduced $(WB - R)$ are plotted against time of heating.

It is evident that during the first 15 minutes most of the reduction has taken place. Actually the ferricyanide reduced at 20 minutes is only 1·5 % more than at 15 minutes (with this amount of sugar present). The 15-minute period was adopted as the standard heating time.

THE STANDARDISATION GRAPHS.

Maltose.

The maltose used was a sample of Kahlbaum's pure maltose which had been recrystallised three times from 60 % alcohol and then dried in warm air. For this specimen I am indebted to Mr S. W. Cole of the Biochemical Institute, Cambridge. When dried *in vacuo* over concentrated sulphuric acid for 2 hours the sugar lost 0·1 % in weight. The optical activity was $[\alpha]_D^{20^\circ}$ + 130·7° using a solution of about 9 %. This falls within the range of published values for maltose monohydrate, although it is somewhat greater than the average given by Tollens [1914] as + 129°–130°.

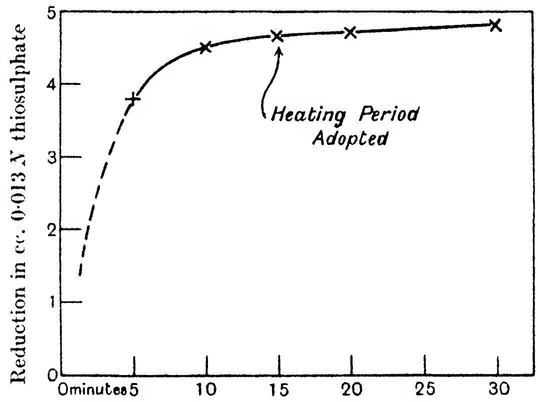


Fig. 2.

Owing to the difficulty of preparing anhydrous maltose, this was not attempted, but the data have been obtained from the hydrate and calculated in terms of the anhydrous sugar by multiplying weights of the hydrate by the factor 342/360.

Maltose solutions of known strength were prepared by weighing out quantities of the sugar and making up to a known volume in a standardised volumetric flask. A set of standardised Ostwald pipettes, from 0·5 to 5·0 cc. capacity was used to measure out portions of these solutions into boiling-tubes containing the standard 5 cc. of solution A, and the amount of ferricyanide reduced after 15 minutes' heating was determined in the way described. These data have been used to construct the standardisation graphs (Fig. 1). The reliability of the procedure may be seen from the fact that the points which were obtained from three different maltose solutions all lie very nearly on the same line. The experimental data on which these points are based are given in an appendix.

In the case of maltose the relationship between ferricyanide reduced and the amount of sugar is a linear one. 1 cc. of $N/100$ thiosulphate is equivalent to 0·414 mg. anhydrous maltose over the entire range and it is therefore convenient to employ this factor for the conversion of reduction values (WB − R) into maltose.

Glucose.

The glucose used was a sample of "Dextrose A.R." (British Drug Houses). It was desiccated *in vacuo* over concentrated sulphuric acid for 48 hours by which time it was found to have attained a constant weight. The specific rotation of this desiccated sugar was $[\alpha]_D^{20^\circ} + 52{\cdot}2^\circ$ in a 10 % solution. Theoretical value for anhydrous glucose $+ 52{\cdot}5^\circ$.

The glucose standardisation data were obtained as for maltose. The points from three solutions lie very nearly in the same line, but the relationship is not linear here as it is for maltose. This difference between the two sugars was investigated. The reduction progress curves for small and large amounts of glucose or maltose have different forms. When plotted as percentages of the reduction after 30 minutes' heating against time of heating, the curves intersect: those for maltose at 15 minutes, for glucose at 10. Consequently after the standard heating (15 minutes) the reduction by maltose is proportional to the maltose added, but this is not so for glucose since at 15 minutes the percentage progress curve for a small amount of sugar lies below the curve for a large amount. For glucose then, reduction values are most conveniently converted into sugar values by reading from a graph. Table I gives figures for constructing this.

Table I. *The glucose standardisation graph.*

Mg. glucose	cc. $N/100$ thiosulphate	Mg. glucose	cc. $N/100$ thiosulphate
0·20	0·58	2·00	~~2·40~~ 5·9
0·50	1·45	2·50	7·38
0·80	2·32	3·00	8·93
1·00	2·91	3·50	10·55
1·50	4·40	3·80	11·53

Note. These figures have been read at convenient intervals from a large scale graph of the experimental data (Fig. 1).

THE STABILITY OF THE ALKALINE FERRICYANIDE (SOLUTION A).

It was thought important to investigate the keeping qualities of solution A in view of the fact that certain authors have suggested that the alkaline ferricyanide solution of the original Hagedorn and Jensen method should not be kept long. One modification of the original method has been proposed in which the ferricyanide and the alkali are made up separately, to be mixed immediately before the estimation (Pattersen, quoted by Cole [1928]). Accordingly lots of solution A were made up at different times and stored in black-jacketed bottles at room temperature, to be compared at a later date. The results are given in Table II for the reducing values found for 5 cc. of a maltose

solution containing 3·86 mg. maltose, using these solutions. The change after the second day is very slight and it must be concluded that storing the solution for 3 months causes practically no deterioration. During the first 2 days of storage, however, there seems to be an appreciable change in the reduction, solution A should therefore be prepared 2 or 3 days before using. The solution A used in the standardisation estimations had been made up 6 to 10 days.

Table II.

Time of storing solution A	Values in cc. 0·01290 *N* thiosulphate: (*a*) Water blank	(*b*) With 3·86 mg. maltose	(*c*) Thiosulphate equivalent of reduced ferricyanide
10 min.	9·59	2·39	7·20
2 hrs	9·61	2·40	7·21
2 days	9·61	2·32	7·29
8 ,,	9·64	2·34	7·30
28 ,,	9·60	2·35	7·25
34 ,,	9·54	2·21	7·33
90 ,,	9·63	2·30	7·33

The effect of dissolved oxygen.

I have found previously[1] that when copper methods are used a considerable error may be involved in estimating the reducing power of sugar solutions with different amounts of dissolved oxygen. Thus, with the Shaffer and Hartman micro-method, two samples of the same sugar solution, one free from oxygen and the other saturated with oxygen, give reducing values differing by as much as 25 %. The lower the concentration of dissolved oxygen, the higher becomes the reducing value obtained. This was shown to be due to the re-oxidation of cuprous to cupric copper by dissolved oxygen in the early stages of the reduction heating.

The Hagedorn and Jensen method (and the present modification) are found to be entirely free from this defect, as is shown in the following data, which were obtained by taking two portions of a solution of maltose, saturating one with oxygen by bubbling the gas through it and washing the other with a stream of pure nitrogen. The reducing values were then determined.

Mg. maltose found in 5 cc. sample.

	(*a*) Free from oxygen	(*b*) Saturated with oxygen
Solution 1	4·05	4·02
,, 2	1·54	1·54

Since the values found are very nearly the same it is evident that there is no appreciable re-oxidation of the ferrocyanide by dissolved oxygen under the conditions of the procedure. It is much safer, therefore, to use a ferricyanide method when dealing with solutions whose oxygen equilibrium may have been disturbed by any previous treatment such as boiling, filtering under reduced pressure, or washing with a gas stream other than air.

[1] Hitherto unpublished.

The effect of the presence of starch.

The present method was worked out primarily for the purpose of following the progress of sugar production during the hydrolysis of starch; it was necessary therefore to find out whether the presence of an excess of starch interfered with the final iodine titration. It is found that although the titration is slightly more difficult in that there is a tendency to overshoot the end-point, a little practice overcomes this difficulty. The stirring must be more vigorous and towards the end of the titration the thiosulphate must be added more slowly. Experiments were done in which known amounts of maltose were added to solutions of soluble starch and amylose of various concentrations. The latter were undialysed and had small reducing powers themselves. Values found for added maltose, subtracting the reduction due to the starch or amylose, were in all cases in close agreement with the amounts added.

Summary.

The range of the Hagedorn and Jensen sugar method has been extended to enable the estimation of about ten times the amount of reducing sugar. The solutions and procedure for the modified method are described and standardisation data are given for glucose and maltose. An important advantage over copper methods is pointed out in the fact that variations in the amounts of dissolved oxygen in sugar solutions do not affect the reducing values arrived at by the ferricyanide method.

In conclusion I wish to express my thanks to Mr S. W. Cole of the Biochemical Institute, Cambridge, for his very generous help and criticism.

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APPENDIX.

The standardisation data.

Water blank values: 9·62, 9·63, 9·625, 9·605, 9·615, 9·61, 9·62, 9·61, 9·62 cc.
Thiosulphate used: 0·01290 *N*.

Mg. glucose	3·71	3·39	3·08	2·49	2·05	1·50	1·25	0·829	0·749	0·622	0·414	0·213	
Ferricyanide reduced*	9·72	7·90	7·11	5·69	4·64	3·42	2·83	1·83	1·69	1·39	0·88	0·46	
(WB − R)	9·68	7·92	7·12	5·70	4·64	3·40	2·83	1·84	1·69	1·37	0·89	0·48	
Mg. maltose	4·86	4·44	3·93	3·86	3·13	2·47	1·96	1·89	1·56	0·980	0·780	0·382	0·197
Ferricyanide reduced*	9·12	8·29	7·40	7·32	5·86	4·65	3·69	3·59	2·92 2·94	1·81	1·46	0·70	0·39
(WB − R)	9·09	8·28	7·39	7·31	5·92	4·66	3·69	3·57	2·92	1·82	1·47	0·71	0·37

* Expressed in terms of cc. of 0·01290 *N* thiosulphate.

XV. THE NATURE OF THE UNSAPONIFIABLE FRACTION OF THE LIPOID MATTER EXTRACTED FROM GREEN LEAVES.

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(Received January 1st, 1929.)

In an investigation now being carried out by E. M. Hume at the Lister Institute, the unsaponifiable matter of the lipoid substances extracted from spinach leaves was being prepared by her to supply the vitamin A in a certain diet. During the course of this preparation we noticed that a white crystalline substance readily separated when this unsaponifiable matter was being taken up with hot alcohol, and we therefore decided to investigate its nature.

Method of preparation. The spinach leaves were dipped for a few seconds into boiling water before drying at 37° under a fan. The preliminary dipping expels air from the tissue and leaves so treated are crisp and green when dry. They were then powdered and extracted with light petroleum (B.P. 40–60°) at the laboratory temperature, being shaken for part of the time of each extraction. The petroleum was removed by evaporation and the residue dried to constant weight, dissolved in ether and a 5 % alcoholic solution of sodium ethoxide added. After standing overnight part of the alcohol was removed by evaporation, water added and the aqueous solution extracted with ether; an orange brown substance was obtained from this ethereal extract. Working in this manner, 2 kg. of spinach gave 230 g. of dried leaf tissue and, after this had been eleven times extracted, each time with 2 litres of light petroleum, 18·4 g. of lipoid substance were separated containing 4·6 g. of unsaponifiable matter. The latter is an orange brown solid giving with concentrated sulphuric acid the blue colour characteristic of carotene. The sterol content was determined by precipitation with digitonin, and was found to be less than 5 %. The sterol was removed from the rest of the unsaponifiable matter and the residue taken up with hot alcohol. On cooling yellowish white plates separated from the orange-coloured solution: after two or three crystallisations they were obtained as glistening white plates, M.P. 68–68·5°. The substance was a hydrocarbon containing 85·1 % C and 14·6 % H (micro-analysis). From these data the substance was identified as the hentriacontane, $C_{31}H_{64}$, originally prepared by Krafft [1882].

If green cabbage leaves are worked up similarly, the crystals separating from the hot alcoholic solution of the unsaponifiable matter melt at 72 to 75° and analysis shows that they contain about 5 % of oxygen. From these

a small amount of crystals melting at 68° was isolated, the melting point of which was unchanged when the crystals were mixed with the hentriacontane obtained from spinach. In cabbage leaves therefore the hentriacontane is accompanied by an oxygen-containing substance which is not present in spinach and which is now the subject of further investigation. The occurrence of these saturated normal hydrocarbons in green leaves is of some interest. A survey of the literature shows that three of them, containing 27, 31 and 35 carbon atoms, have been definitely identified in green leaves. These were originally prepared by Krafft [1882] by heating the barium salts of the fatty acids and reducing the resulting ketones with hydrogen iodide and phosphorus.

$$C_nH_{2n+1}.COO.Ba.OOC.C_nH_{2n+1} \rightarrow C_nH_{2n+1}.CO.C_nH_{2n+1} + BaCO_3$$
$$\rightarrow C_nH_{2n+1}.CH_2.C_nH_{2n+1}.$$

From myristic, palmitic and oleic (or stearic) acids, the acids most commonly occurring in plant fats, the hydrocarbons which would be produced by the above reactions are those containing respectively 27, 31 and 35 carbon atoms.

The following table shows their occurrence in plants.

$C_{27}H_{56}$ Heptacosane M.P. 59·5° C = 85·2 % H = 14·8 %	$C_{31}H_{64}$ Hentriacontane M.P. 68·5° C = 85·2 % H = 14·8 %	$C_{35}H_{72}$ Pentatriacontane M.P. 75° C = 85·3 % H = 14·7 %
	Occurrence	
	Beeswax*	
Leaves of tobacco†‡	Leaves of tobacco†‡	
	Seeds of Ko-Sam§	
	Leaves of *Gymnema sylvestre*‖	
	,, *Grindelia robusta*¶	
	,, *Morinda longiflora***	
	,, *Erodyction*††	Leaves of *Erodyction*††
	,, olive‡‡	Leaves of olive‡‡
	Bark of olive§§	
	Leaves of spinach	
	,, cabbage	

* Schwalb [1886]. † Thorpe and Holmes [1901]. ‡ Mabery [1905]. § Power and Lees [1903]. ‖ Power and Tutin [1904]. ¶ Power and Tutin [1905]. ** Barrowcliff and Tutin [1907]. †† Power and Tutin [1906]. ‡‡ Power and Tutin [1908, 1]. §§ Power and Tutin [1908, 2].

Two of the richest sources of vitamin A are found in the unsaponifiable matter obtained respectively from green leaves and from fish-liver oils and it is interesting that there is a certain parallelism between the constituents occurring in them.

Both contain (1) a highly unsaturated hydrocarbon, (2) products which may be regarded as obtained from the higher fatty acids by processes of condensation and reduction, and (3) sterols. The first of these is represented in the leaf material by the hydrocarbon carotene, containing 11 ethylenic linkages [Kuhn and Winterstein, 1928]: in the liver oil by squalene with six unsaturated linkings (or by similar unsaturated hydrocarbons). Both these hydrocarbons are built up from a number of isoprene units, and show considerable resemblance [Chapman, 1923; Heilbron, Kamm and Owens, 1926]. The second constituent is represented in the leaf by the high normal saturated

hydrocarbons enumerated above, in the liver oil by the batyl, selachyl and chimyl alcohols which are now known to be condensation products of glycerol with the higher fatty alcohols corresponding to stearic, oleic and palmitic acids [Heilbron and Owens, 1928]. Although the available evidence is against the identification of any of these substances as the active agent [Drummond, Channon and Coward, 1925], the existence of a certain similarity of composition in these two materials obtained from such widely different sources, both rich in vitamin A, is not without interest.

We desire to acknowledge our indebtedness to Dr Morgan who carried out the micro-analyses for us, and to the Department of Scientific and Industrial Research for the grants which have made the work possible.

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XVI. THE BIOCHEMISTRY OF DRY-ROT IN WOOD.

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(*Received November 16th, 1928.*)

Introduction.

The biochemical action of the saprophytic and parasitic fungi which cause decay of lignified tissues is very little understood at the present time. It is usually stated in mycological literature that the action of the fungal hyphae is to cause delignification of the lignocellulose complex by removal of the lignin and to leave a residue of cellulose. These results have been mainly attained by the study of microchemical tests on sound and decayed wood, and no systematic investigation has been made on a macroscopic scale.

Microchemical reactions applied to wood as specific tests for the presence or absence of either lignin or cellulose are of little value, as the characteristic reactions (mainly colour changes) are due to small amounts of impurities in the tissue.

It is well known that wood-destroying fungi are rich in enzymes, and as many as a dozen have been reported from a single species. The early investigations of Czapek [1899] showed that greatly increased quantities of hadromal (coniferyl aldehyde) could be extracted from wood that had been attacked by fungi, and he assumed that they contained an enzyme, hadromase, which split a supposed ether-linkage between the hadromal and cellulose. Somewhat similar results were obtained by Zeller [1916] who isolated an enzyme extract from the mycelium of *Lenzites saepiaria* which was found to act on a substrate of the sapwood of *Pinus echinata*, and an alcoholic extract of the residue gave the phloroglucinol reaction. He proposed the name lignase for Czapek's hadromase.

The investigations of Hartig [1900], based on colour changes with microchemical reagents, showed that the parasites *Polyporus vaporarius*, *P. sulphureus*, and the saprophyte *Merulius lachrymans* removed cellulose from wood and left a residue of wood-gum, while *Trametes pini* was found to remove lignin and leave pure cellulose. After the loss of lignin, the middle lamella (which was assumed to be composed of pectin) dissolved and left isolated tracheids. This latter result is obviously incorrect, as we now know that the middle lamella is composed largely of lignin.

The arguments advanced by Hartig and the majority of subsequent

investigators that decayed wood consists of pure cellulose are based upon staining the residue of this wood with zinc chloroiodide—a very unsafe standard. Other workers have also stated that members of the Polyporaceae remove lignin from wood, leaving cellulose. Lindroth [1904] found that the phloroglucinol reaction disappears first in decayed wood and then the Maule reaction also disappears. As the red colour given by wood with phloroglucinol and hydrochloric acid disappears, the colour reactions for cellulose become more intense, until finally pure cellulose is indicated.

Again, Marshall Ward [1897] by the study of colour changes with micro-chemical reagents came to the conclusion that the action of *Sterium hirsutum*, which attacks *Aesculus* (Chestnut), is to destroy the lignin and leave the cellulose. Ward, however, was unable to isolate any enzyme capable of causing delignification.

The evidence for the production of cellulose by *Trametes pini* is founded on stronger grounds than any of the investigations discussed above. Johnson and Lee [1923] found an increase of 15 % in cellulose and a decrease of 30 % in lignin in wood attacked by this fungus.

The first complete examination of decayed wood (Douglas Fir) was made by Rose and Lisse [1917]. Unfortunately the fungus that produced the decay was not identified. There was a marked increase in the solubility of the decayed wood in alkali and a decrease in the amount of cellulose, pentosans and acetyl groups, while the methoxyl content was increased.

From the conflict of evidence on this subject it was considered of interest to carry out an investigation of wood attacked by *Merulius lachrymans*, using modern methods of cellulose analysis. Spruce-wood (*Picea excelsa*) was chosen for the experimental work as its constants are well known and form the requisite standard of comparison for the experimental data. Only completely decayed wood was used to study the action of the fungus.

The action of *Merulius lachrymans* on timber is too well known to need detailed description. The wood loses weight and is converted into the so-called touch-wood, which powders easily to give a brown dust.

It was found that the decayed spruce-wood gave Ditz's test for oxycellulose. A small amount of the wood was suspended in water and methyl orange was added to colour the water yellow. On addition of a few cc. of a saturated solution of sodium chloride, a wine-red colour was developed at once. Sound wood did not give the reaction. A red colour was produced with phloroglucinol and hydrochloric acid. The decayed wood rapidly reduced Fehling's solution, whereas sound wood only brought about reduction after prolonged boiling. Similar results were obtained with decayed beech-wood (hard wood) which had been attacked by *M. lachrymans*. 47·2 % of the decayed wood was soluble in a 5 % solution of sodium hydroxide. The ash-content was 1·07 % and the natural moisture 13·4 %. The technique in general employed was that elaborated by Dore [1920], and in Table I a comparison is made of the values obtained for sound and decayed spruce.

Table I. *Comparison of sound and decayed spruce.*

	Sound wood %	Decayed wood %
Extracted by benzene ...	1·7	1·55
,, alcohol ...	1·6	9·46
,, water ...	2·5	5·43
,, 5 % NaOH	3·8	30·30
Cellulose	55·0	11·90
Lignin	28·0	43·00
Mannan	7·6	0·00
Galactan	0·1	0·00
	100·3	101·64
Ash-content	0·86	1·07
Natural moisture ...	10·0	13·40

(All analyses are calculated on ash-free material dried at 105°.)

An examination of the figures shows that a decrease of 60 % has occurred in the cellulose, while the lignin has increased by 57 %. It was found in the course of the analysis of the cellulose that the lignin in the wood had in some way been altered. When the wood was placed in chlorine gas to chlorinate the lignin prior to its removal by boiling with sodium sulphite solution, instead of the usual golden-yellow colour being formed, the whole mass turned a dull brick-red. A further point in this connection is the complete removal of the hexosans, mannan and galactan. The portion soluble in 5 % sodium hydroxide solution (after removal of the fractions soluble in benzene, alcohol and water respectively[1]) represents, in part at any rate, the hemicellulose components of the wood. These bodies are soluble in dilute caustic alkali and are readily hydrolysed by acids. They contain "uronic" acids of the type of galacturonic acid [Nanji, Paton and Ling, 1925], as well as pentose and hexose residues. It has been shown by O'Dwyer [1926] that the hemicellulose fraction of beech-wood consists of two substances, hemicellulose A, which is precipitated from alkaline solution by acid, and hemicellulose B, soluble in water but insoluble in alcohol. Hemicellulose A contains xylose and 11 % of glycuronic acid, whereas hemicellulose B contains arabinose and 63 % of galacturonic acid as well as small amounts of galactose. Dorée and Barton-Wright [1927] have also shown that two hemicelluloses are present in spruce-wood. That a part at least of the sodium hydroxide-soluble fraction of decayed wood consists of hemicelluloses was indicated by the presence of increased amounts of uronic acids. These on distillation with 12 % hydrochloric acid do not give a quantitative yield of furfural; but the carbon dioxide evolved by the decarboxylation of their carboxyl group affords an exact measure of their amount. The yield of uronic acid from the good wood was 2·688 %, and from the decayed wood 4·84 %; an increase of 82 %. The greater part of the alkali-soluble fraction was precipitated by the addition of acetic acid, and the remainder, after concentration, by adding alcohol [cf. O'Dwyer, 1926]. The increase in solubility of the decayed wood in NaOH solution compared with that of sound spruce

[1] The total of the values found for the benzene, alcohol, water and 5 % sodium hydroxide extract (47·2 %) agrees well with the solubility of the wood in 5 % sodium hydroxide alone (46·71 %), without preliminary extraction with organic solvents and water.

is very high and cannot be entirely accounted for by the presence of hemicelluloses. It is possible that the remainder of the alkali-soluble fraction is composed of the metabolic products of the fungus and the contents of the fungal hyphae.

Comparison of the figures for the methoxyl content of sound and decayed wood shows an increase in the latter of 77 % (sound wood, OMe = 5·44 %; decayed wood, OMe = 9·67 %). A small part of the methoxyl content is located in the hemicellulose fraction [see O'Dwyer, 1928], while the rest occurs in the lignin.

We have obtained evidence (which we hope to publish in a later communication) that the major part of the alcohol extract is composed of lignin. It is possible that it is of the nature of the metalignin isolated by Dorée and Barton-Wright [1927], which has the molecular composition $C_{20}H_{20}O_6$ and is the simplest type of lignin known.

Discussion.

Direct determination of the cellulose and lignin in decayed wood shows decrease of the former and increase of the latter, and estimation of the methoxyl groupings confirms the increase of the lignin. These results show very clearly that no delignification of the wood is brought about by the fungus, but rather that the main attack is confined to the cellulose. The hexosans, mannan and galactan, are also removed, and we would therefore suggest that *M. lachrymans* first attacks these two easily hydrolysed bodies in the course of its metabolic activities and then confines its attack to the cellulose.

It is difficult to account for the increase in the hemicellulose fraction of the decayed wood. The hemicelluloses are readily hydrolysed substances, and the main hydrolytic products are sugars. On the other hand uronic acids are also produced, and it may well be due to the presence of the latter that the fungus does not affect the hemicelluloses.

The influence of the hydrogen ion concentration of the medium on the development of the mycelia of wood-destroying fungi has only been examined in a few cases, but Meacham [1918] found that the first marked deflection in the growth curve occurred at an acidity of $N/350$ HCl and was limited by $N/50$. The hydrogen ion concentration, however, does not appear to be the chief factor determining a desirable medium. According to Zeller, Schmitz and Duggar [1919], no general statement can be made on the relationship between the p_H of the medium and the growth of wood-destroying fungi.

Merulius lachrymans is very sensitive to the presence of acids. Wehmer [1911, 1912] has found that both gallic and tannic acids are markedly toxic to the fungus, and it is for this reason that coniferous (soft) wood is very much more rapidly attacked than a hard wood such as oak. Concentrations of 1–2 % of these acids at once stop all mycelial growth, and coniferous wood can be made resistant to *Merulius* by impregnation with tannic acid. The presence of acids produced by hydrolysis would seriously hamper the fungus in its

attack on the wood, and it may be on this account that the hemicelluloses are not affected. On the other hand, the hydrolysis of the galactan, mannan and cellulose fractions leads to the production of no growth-inhibiting products, but provides the requisite carbohydrate material for active metabolism. With the destruction of the cellulose matrix of the tracheids the touch-wood or tinder-wood condition, which is so typical of wood affected by *M. lachrymans*, is obtained.

Summary.

The effect of the fungus *Merulius lachrymans* (the cause of dry-rot in wood) is to remove the galactan, mannan and cellulose fractions in spruce-wood; the hemicelluloses and lignin are not affected, *i.e.* no delignification of the woody tissues takes place.

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XVII. REDUCTION POTENTIAL, ENERGY EXCHANGE AND CELL GROWTH.

EXPERIMENTS WITH *B. COLI.*

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CONTENTS.

PART I.

Introduction.

So far biological interest in oxidation-reduction potentials has centred mainly on the determination and significance of intracellular r_H. The experimental method has been to inject into cells oxidation-reduction indicators (whose r_H ranges have been determined by Mansfield Clark and his colleagues) and to observe the equilibria obtained between their reduced and oxidised forms.

Needham and Needham [1926] came to the conclusion that "the r_H of the amoeba is probably widely independent of the concentration of oxygen in the external atmosphere" but that an anaerobic protozoon (*Nyctotherus cordiformis*) has "an internal r_H of 19 to 20 under aerobic conditions whilst under anaerobic conditions the latter value changes to r_H 9·5 to 10·5." They considered that "in the facultative anaerobe the oxidation-reduction potential can be adjusted to the environment whilst in the strict aerobe changes in the environment which would lead to such an alteration in the r_H bring about death." Their conclusion that the cell has "a definite, well poised and characteristic equilibrium" was based essentially upon the fact that certain oxidation-reduction indicators became oxidised when injected under anaerobic conditions into the cell in their reduced forms.

Rapkine and Wurmser [1927] have determined the r_H of a number of cells and found them all to be in the zone 19–20. This value was not altered by the injection into the cell of such substances as glucose, sodium pyruvate and succinate. They concluded that "the rate of dehydrogenation is, within wide limits, independent of the concentration of hydrogen donators."

Recently Cohen, Chambers and Reznikoff [1928] have injected twenty-five oxidation-reduction indicators in either oxidised or reduced form into *Amœba dubia* and *Amœba proteus* under controlled oxygen access. They found, contrary to the Needhams, that under anaerobic conditions the amœba was able to reduce *all* the indicators used and that it could not oxidise six of the most easily oxidisable indicators. The phenomena were closely parallel to those observed by Cannan, Cohen and Clark [1926] in their reduction electrode studies on bacterial cultures.

At present, therefore, there is evidence by the Needhams pointing to the existence of a fairly well poised oxidation-reduction system in the cell and there is the opposing evidence of the American workers indicating the existence of an intracellular reduction intensity which increases as the neutralising effect of oxygen is eliminated.

It is difficult to estimate, however, how far r_H terminology can be applied to the living cell. The application of r_H terminology implicitly assumes the existence in the cell of equilibria arrived at almost instantaneously in a homogeneous reversible system. Yet the cell is a heterogeneous system; its enzymes and catalysts are in a heterogeneous condition. From the work of Brooks [1926], Rapkine and Wurmser [1926] and Cannan [1926] it is known, for example, that relatively high reducing potentials can be maintained in the

green cell apparently in the presence of free or molecular oxygen. It is doubtful therefore what significance can be given to the actual value of intracellular r_H; its value (determined by the ratio of reduced to oxidised form of indicator) is a function of many factors, inevitable in a heterogeneous system of catalysts, and to perceive its significance it is necessary to disentangle and estimate the influence of each factor.

Michaelis and Flexner [1928] point out that when a tissue, naturally aerobic, is kept under anaerobic conditions it undergoes reactions which ultimately reach an equilibrium, the final potential obtained being in some way related to the chemical composition of the tissue in its living state. But, granting that such a final equilibrium potential can be determined, it is difficult to see how it can be interpreted to give some idea of the state of the cell under normal conditions; for it is with velocities of change that the normal events of the cell are primarily concerned and these velocities may in many cases be independent of the magnitude of a potential.

We have approached the problem of the relationship of r_H to events in the cell in a different manner from that which has hitherto been adopted.

Quastel and Stephenson [1926] suggested that the facts relating to the action of oxygen on the proliferation of a strict anaerobe such as *B. sporogenes* could be best interpreted by assuming that the cell must attain a certain limiting reduction potential before proliferation could occur. At a potential more oxidising than this the cell would fail to proliferate; below this potential proliferation was possible. This point of view has been confirmed by the recent work of Aubel, Aubertin and Genevois [1928].

The question arose as to whether a facultative anaerobe such as *B. coli* needed a definite r_H range within which to proliferate and this led to the enquiry: does the growth of the cell depend at all on the oxidising or reducing intensity, on the r_H, of the environment? The experiments on *B. sporogenes* seem to show that a particular value of reduction potential is necessary before proliferation of the anaerobe can occur. But is this phenomenon a general one? The following communication constitutes an attempt to answer this question.

Experimental method.

Our experimental method, briefly, has been to determine how far the growth of *B. coli* on synthetic media is affected by changes in the reducing or oxidising power of the media in presence of the cell. The work has been made possible by a knowledge of the reactions brought about by the various constituents of the media in presence of the "resting" or non-proliferating organism.

Throughout the experiments described below, the inorganic medium employed was as follows:

0·4 g. $(NH_4)_2HPO_4$.	0·07 g. $MgSO_4$, $7H_2O$.
0·1 g. NaCl.	Trace $FeSO_4$.
0·1 g. KH_2PO_4.	100 cc. distilled water.

This inorganic medium was usually made up to three times the strength given and kept as stock. When made up into a nutritional medium it was always so diluted that the final strength of salts was that given. The organic acids contained in the media were added in the form of their sodium salts and the final p_H of all media was 7·2. All experiments were done in duplicate, and series of experiments were repeated many times. 5 cc. of each medium, after autoclaving, were sown with 2 drops of a freshly grown tryptic broth culture of *B. coli*. Usually 20-hour growths were used but 6-hour growths are very satisfactory. It is advisable to use young cultures, results with old ones being inconsistent and variable. A heavy sowing, such as that given in 2 drops, was usually found to be necessary. A number of strains of *B. coli* have been used. It is preferable for consistent results to use a strain which gives a good growth, in tryptic broth at 37°, within 6 hours.

In certain experiments, especially when using slowly growing strains of *B. coli*, a trace of an alcoholic extract of marmite was added to the media, controls always being carried out to show that no perceptible growth occurred on the quantity of marmite alone.

Growths were usually examined after 20–40 hours' incubation (aerobic and anaerobic) at 37°. Details of the composition of the various media are given in the tables which summarise the results.

Figures are given in the tables which express relative growths.

The growths were usually estimated by comparison with standard barium sulphate suspensions or nephelometrically. In a number of cases bacterial counts were made but such absolute values were not so important for our purposes as the relative ones. The figures in one table representing relative growths cannot be compared with the figures given in another table, the tabulation being such that the results given in any one particular table can be strictly compared with each other but not with those in any other table.

Synthetic media for anaerobic growth of *B. coli*.

The following three conditions, at least, appear to be necessary before proliferation can occur.

1. The organism must be able to secure energy either by anaerobic decomposition of the substrate (*e.g.* glucose into lactic acid) or by the oxidation of the substrate.

2. The organism must be able to activate the substrate so that the latter is capable of reaction, *e.g.* oxidation or reduction (which it does not necessarily undergo in absence of the cell).

3. The products of oxidation (or decomposition) of the substrate, or the substrate itself, must be capable of entering into the synthetic operations of the organism.

It follows that if molecular oxygen be replaced by some molecule which induces in or around the bacterial cell a state comparable with that which occurs under aerobic conditions and so long as the three conditions stated

above are fulfilled anaerobic growth should occur in presence of this molecule. Now *B. coli* activates nitrates and fumarates as hydrogen acceptors and the conditions enumerated above hold in the systems lactate-nitrate and lactate-fumarate. Anaerobic growth of *B. coli* was found to occur in both media, the lactate giving rise on oxidation to pyruvate which was utilised by the organism [Quastel, Stephenson and Whetham, 1925; Quastel, 1925]. Later it was shown that anaerobic growth occurred, as was anticipated, on glycerol-nitrate, glycerol-fumarate and glycerol-aspartate media and that it did not occur with a strict aerobe, such as *B. alkaligenes* which failed to show any perceptible activating action on nitrates or fumarates [Quastel and Stephenson, 1925; Quastel and Wooldridge, 1925].

In a lactate-fumarate medium, the lactate acts, in presence of the cell, as a hydrogen donator and must be regarded therefore as exerting a reduction potential relative to the cell. Similarly the fumarate must be considered as exerting, in presence of the cell, an oxidation potential. Therefore, although a lactate-fumarate medium, in the absence of the cell, has no observable reducing or oxidising power[1] and gives no potential discoverable at an electrode, it must be considered as exerting an oxidation-reduction potential, dependent on the relative concentrations of the two substances, in presence of the cell. This potential is not observable at an electrode because of the inaccessibility at the electrode of the cell enzymes in combination with their substrates—nevertheless it is clearly indicated by the fact that methylene blue, in presence of the system (lactate-fumarate), will be reduced to a particular value dependent on the relative velocities of reduction by lactate and oxidation by fumarate. Such a value would be as real an indication of the r_H of the medium relative to the cell, as is the intracellular r_H of a cell indicated by the method of microinjection. Now if the growth of *B. coli* is dependent on the r_H of the medium it should be dependent on the relative concentrations of lactate and fumarate in the medium.

Anaerobic growth of *B. coli* on a lactate-fumarate medium (Table I).

Table I.

Growth of *B. coli* anaerobically on media containing lactate and fumarate in varying concentrations. Each column represents a separate medium. Growths examined nephelometrically.

Conc. of lactate × $M/10$	1	1	1	1	1	1	1	1·5	2	3	4	4
Conc. of fumarate × $M/40$	0	0·5	1	1·5	2	3	4	1	1	1	1	2
Relative anaerobic growths after 20 hrs. incubation	0	0·5	1·5	2	2	2	1·5	2·5	1·5	0·5	0	0

The following conclusions may be drawn.

1. Growth increases with increase of lactate concentration until a concentration is reached above which growth diminishes with increase in concentration.

2. Growth increases with fumarate concentration until a maximum is reached. This concentration is about $M/40$. Above this concentration further

[1] On, say, the methylene blue system.

increase of fumarate appears to have but little inhibitory action. High concentrations of fumarate are however definitely inhibitory.

These results are in accordance with those found by Quastel and Stephenson [1925].

The fact that growth increases with increase in lactate and fumarate concentration until maxima in concentrations are reached is easily understood, for the rate of growth must be dependent on the rate of oxidation of the lactate to pyruvate (which is assimilated by the organism) and upon the rate of energy liberation (which is used for synthetic purposes). Such rates must be dependent on the amounts of lactate and fumarate present.

The inhibitory action of lactate at relatively high concentration is not due to any toxic effect of this substance on *B. coli*, for good aerobic growth will occur in lactate-fumarate media where the concentrations of lactate are such that anaerobic growth is markedly inhibited.

The inhibition of growth with increase of lactate concentration may be due, however, to an increased reduction potential being secured within the cell. If this be so it will be expected that increase in fumarate concentration, after attaining a critical value, will balance the inhibitory action of increased concentration of lactate and so result in an increased growth. This does not seem to occur. It may well be argued, however, that the failure of fumarate to balance the lactate effect is due to saturation of the fumarate enzyme with its substrate, so that increase in concentration of the latter does not exert any increased velocity of oxidation and hence any power of neutralising the lactate effect.

This difficulty may be surmounted in the following manner.

In the presence of *B. coli* an equilibrium is established between succinate, fumarate, methylene blue and leucomethylene blue, the equilibrium point being independent of the absolute quantities of succinate and fumarate present [Quastel and Whetham, 1924]. The oxidising action of fumarate is balanced by the reducing action of succinate in a reversible manner characteristic of the usual reduction-oxidation indicators.

If now to a lactate-fumarate medium succinate be added, the r_H of the medium (relative to the cell) must decrease, the fumarate action being opposed by that of the succinate. The evidence points to the same enzyme activating both succinate and fumarate [Quastel and Wooldridge, 1927, 1] so that the difficulty of enzyme saturation does not appear here. Clearly, whatever the concentrations of the two substances, their ratio will determine the reduction potential at their enzyme, *i.e.* in the presence of the cell. Hence by keeping the lactate concentration constant the r_H may be varied by varying the relative proportions of succinate to fumarate.

Anaerobic growth of *B. coli* on a lactate-fumarate-succinate medium.

The results are shown in Table II.

1. Increase in succinate concentration results in a marked inhibition of growth.

2. This inhibition is relatively greater than that produced by increase in lactate concentration.

Table II.

Growth of *B. coli* anaerobically on lactate-fumarate-succinate media of varying compositions.

Conc. of lactate $M/10$	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Conc. of fumarate $\times M/40$	0·5	0·5	0·5	0·5	0·5	0·5	1	1	1	1	1	1	2	2	2	2	2	2	2
Conc. of succinate $\times M/40$	—	0·25	0·5	1	1·5	2	–	0·25	0·5	1	1·5	2	—	0·25	0·5	1	1·5	2	3
Relative anaerobic growths after 20 hours' incubation	2	2	1·7	1·3	1	0·5	3	3	2·2	1·9	1·1	0·5	3	2·9	1·7	1·1	0·8	0·75	0·5

The specific action of succinate.

The inhibiting action of succinate may be shown to be specifically connected with fumarate. Anaerobic growth of *B. coli* in tryptic broth, glucose solution, sodium pyruvate solution or a lactate-nitrate medium is not perceptibly inhibited by the presence of concentrations of succinate which markedly inhibit growth in a lactate-fumarate medium (see Table III). This specific action of succinates supports the view that the oxidising action of fumarate is being opposed by a reducing action of the succinate. It may be shown that the inhibition of growth is not due to toxicity of succinate, in this particular

Table III.

Growth of *B. coli* anaerobically in various media in the presence and absence of succinate. Growths examined after 20 hours' incubation.

	Tryptic broth	Lactate-fumarate	Lactate-nitrate	Glucose $M/20$	Pyruvate $M/10$
Without succinate added	2	2	2	2	2
In the presence of succinate $M/10$	2	0·5	1·5	2	2

medium, for if nitrate be added to such a growth-inhibitive medium, an accelerated growth results. Again a lactate-fumarate-succinate medium exposed aerobically gives a good growth. The effect of the succinate is not due to osmotic action; the same molar concentration of NaCl has no effect on a lactate-fumarate medium. These results are noted in Tables IV and V.

Table IV.

To show anaerobic growths of *B. coli* in lactate-fumarate-succinate media accelerated by the presence of nitrates.

Conc. of lactate $\times M/20$	1	1	1	1	1	1
Conc. of fumarate $\times M/40$	1·5	1·5	1·5	3	3	3
Conc. of succinate $\times M/40$	2	2	2	2	2	2
Conc. of nitrate $\times M/10$	—	0·5	1	—	0·5	1
Relative anaerobic growth after 20 hrs. incubation	0·6	2	2	0·4	2	2

The effects of substances other than succinate on the anaerobic growth of *B. coli*.

So far the evidence would appear to favour the conclusion that the anaerobic growth of *B. coli* in a lactate-fumarate medium diminishes with increased concentration of succinate because the latter is responsible for an increase in reduction potential. The results do not show very clearly, however, that growth is dependent on any ratio of succinate to fumarate. There is remarkably little indication, on the whole, that increase of fumarate concentration neutralises the inhibitive action of increased succinate concentration.

Another explanation of the phenomenon is possible. The same effect of succinate on the anaerobic growth of *B. coli* in a lactate-fumarate medium would obtain if succinates were adsorbed at the fumarate enzyme so that the amount of active fumarate available for hydrogen acceptance would be reduced. Thus if the addition of succinate simply had the effect of competing with fumarate for its enzyme, there would result a diminution in the velocity of oxidation of the lactate. This would not only result in a diminution in the rate of liberation of energy but in a diminution in the rate of production of pyruvate necessary for assimilation. It is easy to see therefore how the presence of succinate would retard cell growth. If this view is true substances other than succinate, perhaps without any reducing action, would bring about the same effect—so long as they competed with either fumarate or lactate for their respective enzymes.

It is known from results on resting bacteria [Quastel and Wooldridge, 1928] that the following substances are among those which compete with lactate for its enzyme: glycollate, α-hydroxybutyrate, oxalate, hydroxymalonate. Malonate competes with succinate for its enzyme. None of these substances is comparable with succinate or lactate for reducing activity at relatively low concentrations; oxalate and malonate have no perceptible reducing action in presence of *B. coli*. Table V shows the effects of these substances on the anaerobic growth of *B. coli* in a lactate-fumarate medium. It will be seen that the substances which compete with lactate or fumarate for their enzymes inhibit growth. The same molar concentration of NaCl has no effect. The same molar concentration of acetate, which does not compete markedly with either lactate or fumarate, has relatively little effect[1].

The evidence points therefore to the action of succinate being just as much due to its competition with fumarate as to its exerting a reduction potential.

This is borne out by another experiment. As indicated earlier *B. coli* will grow anaerobically in a glycerol-fumarate medium. The growth in such a medium takes place with much lower concentrations of fumarate than will allow anaerobic growth in a lactate medium, *i.e.* a smaller number of activated fumarate molecules are necessary for growth on glycerol than for growth on

[1] Significance should not be attached to the results obtained on the addition of acetate to nitrate media since growth of *B. coli* occurs anaerobically in an acetate-nitrate medium.

lactate. (An explanation for this will be suggested later.) It would be possible, if the competition theory were true, that the addition of succinate to a glycerol-fumarate medium, whilst diminishing the total number of activated fumarate molecules, would still leave sufficient of the latter to induce relatively unaltered growth. Experiment (Table V) shows that a concentration of succinate which markedly inhibits growth in a lactate-fumarate medium has but little or no effect in a glycerol-fumarate medium, the amounts of fumarate being in each medium the same.

Table V.

To show the effect of the addition of various substrates to basal media, containing a hydrogen donator and acceptor, upon the anaerobic growth of *B. coli*. Each tube contains 5 cc. of medium consisting of the specified inorganic medium together with the concentrations of H-donator and H-acceptor given in the first column. The substrates are noted at the head of subsequent columns: each being present in $M/20$ concentration. Two drops of an 18-hour broth culture of *B. coli* were inoculated into each tube. The resulting growth after 30 hours' incubation at 37° anaerobically was estimated nephelometrically and is given by the figures in the table. The growths of each series are mutually comparable. ? = doubtful growth.

	Control	NaCl	Acetate	Oxalate	Hydroxy butyrate	Gly collate	Hydroxy-malo nate	Malo nate	Succinate
$M/10$ lactate* $M/20$ fumarate	3	3	2	1	0·25	0·25	1	1	0·5
$M/100$ lactate* $M/100$ nitrate	2	2	2	1·5	1·5	1·5	1·5	0·5	1·5
$M/100$ fumarate* $M/100$ nitrate	2	2	2	1·5	1·5	2	2	?	1
$M/20$ acetate* $M/20$ nitrate	1·5	2	1·5	2	1·5	2	3	0·75	2
$M/50$ glycerol $M/50$ fumarate	2	3	2	4	2	1	3	3	3
$M/50$ glycerol $M/50$ nitrate	3	3	3	4	2·5	2·5	4	1·5	3
$M/20$ glucose	2	2	1	2	2	2	1·5	2	2
$M/10$ pyruvate	2	2	1	2	2	2	3	3	3

* These series contain 0·5 cc. of alcoholic extract of marmite (1/250), control tubes showing no perceptible growth.

Competitive action.

The specific succinate effect seems to be parallel to the effect of carbon monoxide in competing with oxygen for the oxidase system [Warburg, 1926; Keilin, 1927]. The reversibility exhibited by the CO-O_2-oxidase system, is not so easily demonstrable, however, in the succinate-fumarate system. At most we have only had indications that the inhibitive action of succinate is balanced by an increased concentration of fumarate. Further experiment is still needed to establish this finally. The experimental difficulty is that high concentrations of salts undoubtedly inhibit growth by their osmotic effect; so that only a restricted range of concentrations is possible within which to test for reversibility and the range may not be great enough for the purpose. Very low concentrations of fumarate cannot be used for experiment because at these concentrations a small increase of fumarate will, in the absence of succinate, bring about an appreciably increased rate of growth.

The competitions between succinate and malonate and between lactate and oxalate at their respective enzymes are reversible [Quastel and Wooldridge, 1928], the inhibition of reduction by a competing molecule such as malonate or oxalate not being directly proportional to the concentration of the latter but taking place at a rate which would be anticipated if adsorption occurred. Eadie [1928], who has confirmed the observation [Quastel and Whetham, 1925] that the velocity of reduction of methylene blue by a donator in presence of *B. coli* follows the logarithmic relationship

$$v = k + k' \log C,$$

(where k, k' are constants, C is concentration and v is velocity), concludes that this relationship is best interpreted on the basis of adsorption of the substrate at its enzyme.

It would seem a reasonable argument, in face of the evidence, that the succinate effect is due more to competitive adsorption at the fumarate enzyme than to the succinate exerting a reduction potential effect. The matter has been put to further test by investigating the action of succinates and other substances on a variety of media capable of supporting anaerobic growth. Table V summarises the results. It will be observed that inhibition of growth occurs where direct competition of the additional substance with a substrate of the medium is known to occur [Quastel and Wooldridge, 1928].

Conclusions on the action of succinate.

Part, at any rate, of the inhibiting action of succinate may be attributed to its competing with fumarate for its enzyme. There is left the possibility that some of the action is due to an increased reduction potential. It may be considered that the competition itself is synonymous with the production of an increased reduction potential owing to the diminution in number of activated fumarate molecules and hence in the velocity of oxidation. The intracellular r_H would indeed change to a more negative (reducing) value if the velocity of oxidation by fumarate within the cell were to decrease—assuming no marked poising action within the cell. But the same effect would occur by competition with the lactate molecules; here, in contrast to fumarate, the intracellular r_H would become more positive owing to a diminution in the velocity of reduction. In both cases a diminution of growth occurs. It may appear that there is just one value of intracellular r_H at which the rate of proliferation is greatest; that it diminishes as the r_H becomes more oxidising. But in presence of oxygen when presumably the r_H is still more positive the rate of growth is increased. Hence it becomes difficult, if not impossible, to relate the growth of the cell directly to its internal r_H.

PART II.

It is desirable now to consider the effects of the addition to the basal medium of a substance, normal to tissues, but independent of the cell or its enzymes for its reducing action. Such a substance is glutathione and the experiments to be described show the action of glutathione, cysteine and thioglycollate on the growth of *B. coli*. Details of the experimental technique will be found in Part I.

EFFECTS OF CYSTEINE, GLUTATHIONE AND THIOGLYCOLLATE ON THE GROWTH OF *B. COLI* ON A LACTATE-FUMARATE MEDIUM.

(A) *Anaerobic* (see Table VI).

It will be seen that these —SH compounds[1] affect the rate of anaerobic growth markedly. There is a considerable diminution in the growths, these results being clearly observed after 30–40 hours' anaerobic incubation.

(B) *Aerobic* (see Table VI).

The growths under aerobic conditions are, on the other hand, after 20–40 hours' incubation, at least as profuse as, and often greater than, the growths in the media containing no —SH.

Table VI.

To show effects of —SH compounds on aerobic and anaerobic growth of *B. coli* on lactate-fumarate media at p_H 7·2. Growths were examined nephelometrically after 40 hours' incubation. The —SH compounds were sterilised separately by steaming.

Conc. of lactate × *M*/20 ...	1	1	1	1	1	1	1
Conc. of fumarate × *M*/40 ...	1	1	1	1	1	1	1
Conc. of cysteine × *M*/156 ...	—	0·5	1	—	—	—	—
Conc. of glutathione × *M*/250	—	—	—	0·5	1	—	—
Conc. of thioglycollate × *M*/92	—	—	—	—	—	0·1	0·2
Relative growths aerobic	2·5	5	3	4	4	2·5	3·5
Relative growths anaerobic	2	0·7	0·5	0·8	0·6	1·2	0·5

To account for this phenomenon it might be suggested that under aerobic conditions all the —SH is oxidised by molecular oxygen, so that there is no —SH left to give a high reduction potential. The maintenance in the cell of this potential might account for the diminution under anaerobic conditions in the rate of proliferation of the organism.

QUANTITY OF —SH PRESENT AFTER GROWTH UNDER AEROBIC AND ANAEROBIC CONDITIONS.

To test this view the amounts of —SH left in the medium after growth under aerobic and anaerobic conditions were estimated by titration with dilute iodine solution (Table VII).

In almost every case it was found that the amount of —SH left in the medium under aerobic conditions was equal to, and often greater than, the amount left under anaerobic conditions. Thus, in spite of the fact that there

[1] It is very convenient to speak of —SH as though it were a separate entity.

was present in the medium sufficient —SH to give a high negative reduction potential [Dixon and Quastel, 1923; Michaelis and Flexner, 1928], aerobic growth proceeded apparently unaffected whilst anaerobic growth was markedly inhibited.

Table VII.

To show quantities of —SH (estimated by titration with $N/200$ iodine solution) present in lactate-fumarate —SH media after 40 hours' incubation under aerobic and anaerobic conditions.

Lactate-fumarate media	Relative growths anaerobic	cc. $N/200$ iodine required for 5 cc. medium	Relative growths aerobic	cc. $N/200$ iodine required for 5 cc. medium
Exp. 1.				
Without —SH	2·6	0·1	3·7	0·1
With cysteine initial conc. $M/312$ ...	1·0	1·5	5·5	1·6
With glutathione initial conc. $M/250$	1·2	2·2	5·7	1·6
Exp. 2.				
Without —SH	2·0	0·1	2·5	0·1
With cysteine initial conc. $M/156$ ...	0·5	0·85	3·0	1·15
With glutathione initial conc. $M/250$	0·6	1·4	4·0	1·65

The fact that aerobic growth obtains under such conditions is reminiscent of the work of Brooks, Rapkine and Wurmser, and Cannan, which shows that high reduction potentials can occur within the green cell apparently in the presence of free or molecular oxygen. The fact, too, reminds us of the experiments of Hopkins [1925] on the oxidation of protein in presence of glutathione. Throughout the oxidation, even when the velocity of the latter was at its greatest, a small but definite concentration of —SH was maintained. As Hopkins said, the maintenance of this concentration was determined by the relative velocities of reduction of —S.S— by the protein and of oxidation of —SH by oxygen.

Effects of nitrates and oxygen.

If to a lactate-fumarate medium containing —SH, which therefore only feebly supports the anaerobic growth of *B. coli*, there be added nitrates, an accelerated growth of the organism is observed (Table VIII).

Table VIII.

To show the effects of nitrate on anaerobic growth of *B. coli* in lactate-fumarate —SH media

Conc. of lactate × $M/20$	1	1	1	1	1
Conc. of fumarate × $M/40$	1	1	1	1	1
Conc. of cysteine × $M/156$	—	0·5	1	0·5	1
Conc. of nitrate × $M/1·7$	—	—	—	0·5	0·5
Relative growths after 40 hours' anaerobic incubation	2·0	0·7	0·5	2·1	1·9

In the case of aerobic growth on the —SH media, there is usually a lag in the initial rate of growth which is dependent upon the amount of —SH initially introduced into the medium. But the lag disappears after 30 hours' or less incubation and then there results a very profuse growth. This lag we attribute to the medium being essentially anaerobic in the presence of excess

of —SH; as soon as the excess is removed by oxidation, molecular oxygen becomes freely available to the cells and aerobic growth proper commences.

The quantity of —SH measured after 30–40 hours' incubation does not diminish after further incubation either aerobically or anaerobically; rather does it tend to increase.

The aerobic growth of *B. coli* on the —SH media is not due simply to a growth on the —SH compounds introduced. Control experiments show that the growth on these substances in the concentrations used is small compared with the growths obtained in the lactate-fumarate —SH media. Thioglycollic acid[1], upon which the growth of *B. coli* is most feeble, has similar effects, aerobically and anaerobically, to glutathione.

Interpretation of the effects of —SH.

The fact that aerobic growth is unaffected and anaerobic growth is greatly diminished by the presence of —SH in a lactate-fumarate medium, and that in both cases the same amount of —SH is maintained, points to the conclusion that the ability of the cell to proliferate cannot be directly related to the reduction potential of the medium—if by reduction potential is meant the potential secured by the electromotively active substance present.

Now it has been shown by Kendall and Nord [1926] and by Michaelis and Flexner [1928] that the potential of cysteine at an electrode is markedly affected by traces of free oxygen. What, then, are we to consider the potential of the media which we have employed?

It is difficult to apply r_H terminology to such electromotively unbalanced systems as these. It can be argued that the —SH present in the medium maintains within the cell a reduction potential which becomes more positive in the presence of oxygen than in the presence of fumarate. Proliferation presumably would only occur normally at the more positive value. But if proliferation depends essentially on the maintenance of this more positive potential, why should anaerobic growth of *B. coli* occur on glucose? Here reducing conditions are so intense that, as is well known, hydrogen is evolved. Consideration of potential alone seems to give no satisfactory interpretation. This seems to be inevitable, for the rate of growth necessarily depends upon velocity relations, and only in so far as reduction potentials affect relative velocities can we expect to connect rates of growth with potential measurements.

The question of intracellular hydrogen carriers.

When *B. coli* grows anaerobically on a lactate-fumarate medium, by what means is the hydrogen transferred from the lactate to the fumarate? The two enzymes governing the activation of lactate and fumarate appear from the work on resting bacteria to be distinct from each other[2].

[1] To perceive the effects of these —SH compounds only relatively small quantities must be used.

[2] Some "overlapping" occurs in the case of the lactic and succinic enzymes when these enzymes are studied with normal *B. coli* [Quastel and Wooldridge, 1927, 2].

The enquiry resolves itself into the following problem: will a hydrogen donator react with a hydrogen acceptor in the presence of their specific enzymes but in the absence of any intermediate hydrogen carrier?

Now if such a reaction could occur in the absence of a carrier we would expect that a hydrogen donator, or acceptor, in the presence of its enzyme would be electromotively active, *i.e.* it would give a potential at an electrode in a manner similar to —SH or methylene blue. The experiments bearing on this point do not, however, indicate that a hydrogen donator in the presence of its enzyme has a behaviour characteristic of an electromotively active system. Indeed the experiments of Kodama [1926] on hypoxanthine in presence of xanthine oxidase show that no such potential is produced. Keilin has been able to demonstrate that cytochrome plays the part of hydrogen carrier between oxygen activated by oxidase and hydrogen donators activated by dehydrases. It seems equally possible that in the cell of *B. coli* a carrier transfers hydrogen from the activated donators to the activated acceptors. It remains for experiment[1] finally to decide this, but in the meantime we feel justified in proceeding on the assumption that such intracellular hydrogen carriers are necessary. The scheme is represented in diagrams I and II.

Active Centres	Activated Hydrogen→ Donators	H $\xleftrightarrow[\text{carrier(s)}]{}$	Activated Hydrogen Acceptors	Active Centres

I.

Lactic Dehydrase	Lactate→	H ←‑‑‑ carrier(s)	↗Oxygen →Fumarate ↘Nitrate	Oxidase Fumarate enzyme Nitrate enzyme

II.

The carrier[2] is presumably of the usual oxidation-reduction type. Let its oxidised form be represented by A and its reduced form by AH_2. Then since it is diffusible in the cell (otherwise hydrogen carriage would be impossible) the level of oxidation-reduction potential in the cell will be determined by the ratio $[A]/[AH_2]$. This ratio will be determined by the relative velocities of the reducing and oxidising processes within the cell. Now the rate of oxidation of a hydrogen donator, say lactate, will be determined by the concentration of A and the rate of reduction of a hydrogen acceptor, say fumarate, by the concentration of AH_2. The rates will, of course, be dependent also on the concentrations of donator and acceptor but we may assume that the cell enzymes are saturated with regard to their substrates, so that variation of substrate concentration above their saturation points will not affect velocities.

The growth of *B. coli* will be dependent on at least two factors:

(*a*) the rate of production of pyruvate from lactate,

(*b*) the rate of liberation of energy by oxidation.

[1] Such experiments are now being carried out.

[2] There may be a number of carriers but this does not affect the discussion.

These rates will be determined by the value [A] and hence by the oxidising and reducing processes present. If the value [A] be diminished by the introduction into a cell of another reducing factor, say —SH, the proliferation of the cell will be affected through changes in the rates of pyruvate and energy production. If the rate of reduction of A by —SH is greater than the rate of oxidation of AH_2 by the acceptor, the value [A] will decrease with a consequent decrease in the rate of proliferation.

This, we suggest, is a possible explanation of the effects of —SH on the aerobic and anaerobic growth of *B. coli* on a lactate-fumarate medium. It is known from work on the resting organism that the rate of oxidation of leucomethylene blue by oxygen is much greater than by fumarate. Hence we may expect the rate of oxidation of AH_2 to be greater with oxygen than with fumarate. If therefore the amount of —SH in the cell remains constant, the value of [A] will be greater in the case of oxygen than in the case of fumarate. This value may be sufficiently large in the case of oxygen[1] to allow the proliferation of the cell to proceed unaffected, but in the case of fumarate too small for normal growth.

Energy requirements of the cell.

The rate of growth, as has been stated, depends on at least two rates, those of production of pyruvate and of energy. Now it is possible that there may be two molecules, each capable of giving rise to pyruvate, but whose energy productions per molecule may differ from each other considerably. An example of a pair of such molecules is glycerol and lactate. The yield of energy per molecule in breaking down to pyruvic acid is shown in the following equations:

$$\begin{aligned} &CH_3.CH(OH).COOH + COOH.CH{:}CH.COOH \\ &\qquad = CH_3.CO.COOH + COOH.CH_2.CH_2.COOH + 14 \text{ Cal.} \\ &CH_2(OH).CH(OH).CH_2OH + 2COOH.CH{:}CH.COOH \\ &\qquad = CH_3.CO.COOH + 2COOH.CH_2.CH_2.COOH + 50 \text{ Cal.} \end{aligned}$$

It is easily seen that if the rate of oxidation of glycerol is small compared with that of lactate the rate of energy production by glycerol may still be the same as that by lactate, and, so long as the pyruvate produced in both cases is sufficient for the organism's needs, the rates of proliferation on the two substances may be the same.

This means that if the concentration of A in the cell diminishes to such a small value that normal proliferation with lactate will not occur it may still be sufficiently large for a normal proliferation with glycerol. It has been stated earlier that, actually, anaerobic growth of *B. coli* in a glycerol-fumarate medium takes place at a lower concentration of fumarate than in a lactate-fumarate medium. We expect therefore that the presence of —SH in the cell

[1] We are informed by Keilin that the presence of —SH seems scarcely to affect the rate of oxidation of reduced cytochrome by oxygen.

will not affect the anaerobic growth on a glycerol-fumarate medium to as great an extent as on a lactate-fumarate medium.

Experiment confirms this (Table IX).

Table IX.

To show absence of effect of a small concentration of glutathione on growth of *B. coli* in a glycerol-fumarate medium.

Conc. of glycerol × $M/20$	1	1
Conc. of fumarate × $M/20$	1	1
Conc. of glutathione × $M/250$	—	1
Relative growths after 16 hours' anaerobic incubation	2·0	2·5

PART III.

Let us now consider the effects of addition to the basal medium of a third substance—formic acid. This compound differs from succinic acid in being independent of the lactate and fumarate enzymes and from cysteine in requiring an enzyme to bring about its activation. It is specially useful for our purpose for not only is it the most powerful hydrogen donator in presence of *B. coli* (with the exception of the sugars) but it is inert as a nutritional source of carbon and its energy of oxidation is nearly that of hydrogen itself. Formates are, indeed, the most powerful source of active hydrogen so far as *B. coli* is concerned, and if the rate of proliferation of this organism is to be related to the presence in a medium of an active source of hydrogen, the inclusion of formates would be expected markedly to affect proliferation.

Anaerobic growth of *B. coli* on a lactate-fumarate-formate medium (Table X).

The effects of relatively small quantities of formates on the anaerobic growth of *B. coli* on a lactate-fumarate medium are very striking. There is a greatly accelerated growth which seems to reach its maximum when the formate concentration is about $M/40$. Above this concentration, increase of formate tends to diminish the rate of growth. Now this effect of formates is not only to be observed in a lactate-fumarate medium but in a medium composed of fumarate alone, *i.e.* with no lactate present. The growth in a fumarate-formate medium is considerably less than in a lactate-fumarate-formate medium for the same concentration of formates (see also Table XI).

Effects of formates on anaerobic growth of *B. coli* on lactate-malate and lactate-aspartate media.

It was shown by Quastel and Stephenson [1925] that anaerobic growth of *B. coli* on lactate-malate or lactate-aspartate media is doubtful or absent. Examination of the results given in Tables XII and XIII shows the powerful accelerating effects of formates on the anaerobic growth of *B. coli* in these media.

Now it seems very probable that the effect of formate is connected with its reducing activity, but to test this we have compared formate with a closely allied substance—formamide.

Table X.

To show effects of varying concentrations of formate on the anaerobic growth of *B. coli* in a lactate-fumarate medium. 17 hours' incubation. Details of all experimental technique in Part I.

Conc. of lactate × $M/20$	1	—	—	1	1	1	1	1	1	1	1	1	1·5	2	1	—	1	1	—
Conc. of fumarate × $M/20$	—	1	—	1	1	1	1	1	1	1	1	1	1	1	—	1	1	—	1
Conc. of formate × $M/40$	—	—	1	—	0·1	0·3	0·5	0·7	1	2	3	5	—	—	0·5	0·5	1	1	1
Relative growths	0	0	0	1	3	5	5	6	6	3	2	0·5	1·5	1·5	0	2	6	0	2

Table XI.

Effects of formates on anaerobic growth of *B. coli* on lactate-fumarate media giving relative growths and relative bacterial counts*. 16 hours' incubation.

Conc. of lactate × $M/20$	1	1	—	—	1	1
Conc. of fumarate × $M/20$	—	—	1	1	1	1
Conc. of formate × $M/40$	—	1	—	1	—	1
Relative growths	0	0	0	?	1	3
Relative bacterial counts	22	16	40	80	240	1080

* The bacterial counts (which were done in duplicate) were made by plating out two loopfuls of the medium diluted in sterile saline (1/200). These it must be remembered are viable counts.

Table XII.

To show effects of formates on anaerobic growth of *B. coli* on lactate-malate media. 40 hours incubation.

Conc. of lactate × $M/20$	1	1	1	—	—	1	—
Conc. of malate × $M/20$	—	1	1	1	1	1	1
Conc. of formate × $M/40$	1	—	1	—	1	2	2
Relative growths	0	0	4	0	0	4	2

Table XIII.

To show effects of formates on anaerobic growth of *B. coli* on lactate-aspartate media. 40 hours' incubation.

Conc. of lactate × $M/20$	1	1	1	—	—	—
Conc. of aspartate × $M/20$	1	1	1	1	1	1
Conc. of formate × $M/40$	—	0·5	1	—	0·5	1
Relative growths	0·5	3	3	0	?	?

Activation and effects of formamide.

Formamide at low concentrations is not a powerful donator of hydrogen. In a previous communication [Quastel, 1926] it was considered that formamide had a strong reducing activity (on methylene blue in presence of *B. coli*) even at very low concentrations. Formamide purified according to the method of Willstätter and Wirth [1909] always exhibits such activity at low concentrations. If this preparation of formamide, however, be frozen at $-8°$, allowed partially to thaw and the liquid portion discarded, the solid portion shows a diminished reducing activity. By repeating this operation several times a specimen of formamide may be obtained which shows but little reducing activity at low concentrations ($M/500$). At appreciably greater concentrations the formamide again becomes highly reducing, but it is probable that much of this activity is due to traces of formates still remaining in

the formamide[1]. Experiment shows that formamide does not inhibit reductions of methylene blue due to formate, *i.e.* it does not appear to be accessible to the formate enzyme.

The purest form of formamide which we were able to prepare was used in the experiments whose results are given in Table XIV. No acceleration in the anaerobic growth of *B. coli* was produced by formamide at concentrations in which formates produced powerful effects.

Table XIV.

To show relative effects of formate and formamide on anaerobic growth of *B. coli* in lactate-fumarate media. 18 hours' incubation.

Conc. of lactate × $M/20$	1	1	1	1	1	1	1	1	1	1
Conc. of fumarate × $M/40$	1	1	1	1	1	2	2	2	2	2
Conc. of formate × $M/40$	—	1	2	—	—	—	1	2	—	—
Conc. of formamide × $M/40$	—	—	—	1	2	—	—	—	1	2
Relative growths	1	3	3	1	1	1	5	5	1	1

Anaerobic growth of *B. coli* on lactate-fumarate media at varying initial hydrogen ion concentrations.

When growth occurs in a lactate-fumarate medium containing formates, the media usually become more alkaline owing to the oxidation of the formates. Grey [1914] has made use of the fact that formates are easily broken down by *B. coli* with liberation of alkali in his experiments on the fermentation of sugars. Now it seems conceivable that the accelerating action of formates noted above is due to their oxidation with resulting increased p_H and possibly therefore increased rate of proliferation.

This was tested by growing *B. coli* anaerobically in the presence and absence of formates on lactate-fumarate media, whose initial p_H ranged from 6·0 to 8·0 (Table XV).

Table XV.

To show anaerobic growth of *B. coli* on lactate-fumarate media of varying initial p_H, in the absence and presence of formates. 18 hours' incubation.

Conc. of lactate × $M/20$	1	1	1	1	1	1	1	1	1	1	1	1
Conc. of fumarate × $M/40$	2	2	2	2	2	2	2	2	2	2	2	2
Conc. of formate × $M/40$	—	1	—	1	—	1	—	1	—	1	—	1
Initial p_H	6·0	6·0	6·4	6·4	6·8	6·8	7·2	7·2	7·6	7·6	8·0	8·0
Relative growths	0	0	0·9	2·4	1	1·7	0·7	2·1	0·8	1·7	0·7	1·5

It will be seen that the anaerobic growth of *B. coli* was not accelerated by increase in p_H between the range 6·4 and 8·0. The presence of formates always accelerated the growth in this range. It seems scarcely conceivable, therefore, that the formate effect is simply an effect due to change in hydrogen ion concentration.

[1] It is worth noting that the bacterial test for formate (by reduction of methylene blue in presence of *B. coli*) is more sensitive than any chemical test for formate with which we are acquainted.

Again it should be remembered in this connection that very good growth of *B. coli* occurs anaerobically on glucose and glycerol-fumarate media; and in these media growth is always accompanied by an increase in hydrogen ion concentration[1].

Effects of formates on the anaerobic growth of *B. coli* on glucose and sodium pyruvate media.

Formates at concentrations which markedly accelerate growth in lactate-fumarate media have no perceptible action on the anaerobic growth of *B. coli* in glucose and sodium pyruvate. But relatively high concentrations of formates have a definite inhibiting action (see Table XVI).

Table XVI.

To show effects of formates on anaerobic growth of *B. coli* in glucose and pyruvate media. 18 hours' incubation.

Conc. of glucose × $M/200$	1	1	1	1	—	—	—
Conc. of pyruvate × $M/40$	—	—	—	—	1	1	1
Conc. of formate × $M/40$	—	1	2	4	—	1	4
Relative growths	4	4	4	1	4	4	1·5

Now it is well known that glucose on breakdown by *B. coli* produces formates, so that perhaps the lack of effect on the addition of small quantities of formate to a glucose medium is not surprising. On the other hand, the absence of formate action may be real, *i.e.* formates may definitely have no accelerating action. We have investigated the action of formate on glucose with the latter at a concentration of $M/1400$ and still obtained no accelerating action.

Fermentation of pyruvic acid by *B. coli*.

Investigation of the products formed from sodium pyruvate when a suspension of *B. coli* is placed in a solution of the latter shows that formates are produced in abundance. An examination of the products formed immediately after the pyruvic acid has been fermented indicates that the yield of the lower fatty acids and lactic acid does not account for all the pyruvic acid fermented. It has now been found that another substance is produced during the fermentation. This substance does not give the nitroprusside or guaiacol tests for pyruvic acid, but if a solution of it be heated in presence of a little alkali it breaks up to yield a solution once more giving the nitroprusside and guaiacol reactions. This phenomenon is being investigated further by one of us (W. R. W.).

The production of formate from pyruvate on fermentation by *B. coli* may be a reason why the former in low concentration does not accelerate the growth of *B. coli* on a pyruvate medium.

[1] Formates, as is shown later, do not perceptibly accelerate growth on a glycerol-fumarate medium. Such an acceleration would be expected if the formate effect was due to intracellular increase in p_H.

Interpretation of the formate effect.

There is little doubt that the action of formates on growth in the lactate-fumarate medium is connected with their reducing action in presence of the cell; the growth is accelerated. Yet in the case of cysteine or glutathione, where again there was little doubt that the effects were connected with the reducing action of —SH, the growth was inhibited. It appears to be quite certain therefore, as has already been concluded, that the rate of proliferation of *B. coli* cannot be directly related to the reducing or oxidising action of the medium in presence of the cell. Nevertheless formates at relatively high concentrations do inhibit growth and this inhibition is not due to toxicity as can be shown by introducing nitrates into the medium when growth is found to be accelerated. Formate shares this inhibitory action at relative high concentration with similar high concentrations of lactate, succinate or —SH, and it seems quite possible that this action is due to a high reduction potential being set up which may be neutralised by the presence of nitrates or of oxygen.

Formates are therefore abnormal in exerting a marked accelerating action at relatively low concentrations on the growth of *B. coli.* It should be possible to account for this phenomenon on the views which have been put forward to explain the results observed with succinate and with —SH.

This can quite easily be done when it is remembered (1) that the energy of oxidation of formate is nearly that of hydrogen itself,

$$H.COOH + COOH.CH\!:\!CH.COOH$$
$$= CO_2 + COOH.CH_2.CH_2.COOH + 28 \text{ Cal.}$$

and (2) that formates are extremely active donators of hydrogen (in presence of *B. coli*) even at low concentrations.

It will be recalled that in interpreting the action of —SH on lactate-fumarate media and glycerol-fumarate media the suggestion was made that although the —SH brought about decreased rates of oxidation of lactate and glycerol, the energy production in the latter case was sufficiently large, but in the former case not sufficiently large, to allow the normal rate of proliferation to occur. It was assumed that sufficient pyruvate was produced for the organism's needs. The same suggestion may be made to account for the effects of formates. The formate may indeed have the effect of lowering the rate of oxidation of lactate, but, so long as this is not lowered so far that there is insufficient pyruvate produced for synthesis, the greatly increased energy production due to the oxidation of the formate itself would bring about the increased rate of growth. Thus formates should exhibit a maximum effect in their accelerating action, as is actually observed in experiment.

This explanation will account also for the accelerating action of formates on the anaerobic growth of *B. coli* on lactate-malate and lactate-aspartate media. Both malates and aspartates are hydrogen acceptors in presence of *B. coli*, though feeble compared with equal concentrations of fumarate. The fact that formates induce anaerobic growth on fumarate or malate alone is

explained when it is recalled that both fumarate and malate serve as nutritional sources of carbon in the presence of air or of nitrates. Presumably fumarate and malate can undergo self oxidation and reduction each giving rise to succinate and pyruvate (by decarboxylation of oxalacetate) and the presence of formates yield sufficient energy by their oxidation to make anaerobic growth possible. Formates will not induce anaerobic growth of *B. coli* on succinate, or lactate or glycerol or on mixtures of these substances.

This view can be put to further test. Clearly if the formate action is simply connected with the energy of oxidation of formate, the addition of a formate to a glycerol-fumarate medium should not have the same accelerating action as is observed in a lactate-fumarate medium. This is found to be the case (see Table XVII).

On these lines too we may account for the apparent absence of accelerating action of formates on growth in glucose and pyruvate media.

Table XVII.

To show the effects of formates on anaerobic growth of *B. coli* on glycerol-fumarate media. 18 hours' incubation.

Conc. of glycerol × $M/20$	1	1	1	—	—	—	1	1	1
Conc. of fumarate × $M/20$	—	—	—	1	1	1	1	1	1
Conc. of formate × $M/40$	—	0·25	1	—	0·25	1	—	0·25	1
Relative growths	0	0	0	0	1	1	3	3	3

A comparison of *B. coli* with *B. sporogenes*.

It is interesting at this stage to compare these two organisms.

Growth of *B. sporogenes* appears to depend on the acquirement by the cell of a reduction potential of such a value that certain oxidations can be eliminated[1]. But it should be emphasised that it is not the magnitude of the potential which is of great significance; it is the fact that the substance responsible for this potential can reduce quickly enough to prevent certain oxidations (say by oxygen) bringing about a disturbance in the sequence of events which results in proliferation. If we could imagine the cysteine in a medium capable of supporting the growth of *B. sporogenes* to be entirely metal-free, we should expect no proliferation to occur in spite of the fact that metal-free cysteine has a high reduction potential [Harrison and Quastel, 1928]. The presence of —SH in a cell might prevent the oxidation by molecular oxygen of some grouping or constituent vital for the normal sequence of reactions in the cell. But this protection would depend not so much on the potential secured by the —SH as on the velocity of reduction due to the sulphydryl compound. The two, potential and velocity, are connected with each other (being both dependent on the concentration)—but the magnitude of the velocity will depend on the nature of the body catalysing the activity of the —SH.

Anaerobic growth of *B. coli* is inhibited in a lactate-fumarate medium by the presence of —SH. This we have suggested is due to the decrease in velocity

[1] Thus —SH (*e.g.* cysteine) will induce growth of *B. sporogenes* even under aerobic conditions.

of oxidation of lactate due to the presence of —SH. In effect the —SH competes with the lactate for the reduction of the fumarate. A definite velocity of oxidation of lactate—or of glycerol—is necessary for the proliferation of *B. coli*, and all the effects of succinate, —SH, and formate can be consistently interpreted on this view. The anaerobic growth of *B. coli*, as has been stated in earlier papers [Quastel, Stephenson and Whetham, 1925; Quastel and Stephenson, 1925] is not in any way fundamentally different from its aerobic growth[1].

Summary and conclusions.

We have examined the effects of three substances, succinate, cysteine (with which are associated glutathione and thioglycollate) and formate on the anaerobic growth of *B. coli*, particularly in a lactate-fumarate medium. The first substance is known to be specifically associated for its activation with the fumarate enzyme; the second is independent of the cell for its reducing action and can act in a homogeneous phase; and the third is dependent on the cell for its reducing action but is independent of the lactate and fumarate enzymes.

It is now shown that:

(*a*) Succinate inhibits the anaerobic growth of *B. coli* in the lactate-fumarate medium. The effect may be accounted for by its competition with fumarate for its enzyme but it is possible that part of the effect may be attributed to its securing a high reduction potential within the cell.

(*b*) Cysteine (or —SH) inhibits the anaerobic growth of *B. coli* in the same medium and the effect is accounted for by its inhibiting action on the velocity of oxidation of the lactate. This may be considered a true reduction potential phenomenon but it is emphasised that the effect is due rather to the speed of reduction by the —SH than to the magnitude of the reduction potential it secures.

(*c*) Formate at relatively low concentrations (when it is, however, highly reducing) increases the rate of proliferation of *B. coli* in a lactate-fumarate medium and this is accounted for by the rapid oxidation of formate with high energy yield. All the other evidence given supports these conclusions.

We may now attempt to answer the question we have set ourselves: does the growth of the cell depend on the reduction or oxidation intensity, on the r_H, of the environment? We feel justified in concluding from these experiments on succinate, —SH, and formate that the rate of growth of *B. coli* is not dependent on the oxidation or reduction intensity of its environment. Relatively high concentrations of these substances do produce, however, a similar inhibitory action on the anaerobic growth of *B. coli* and

[1] It is worth emphasising that oxygen is simply one of a number of hydrogen acceptors, each possessing its characteristic properties and each important from the point of view of cellular metabolism. It is a study of all hydrogen acceptors, which induce growth, not only that of oxygen, which is essential if a true picture of the normal life of the cell is to be obtained.

it is possible that with these concentrations certain limiting conditions are secured above which growth cannot take place. These conditions are associated probably more with kinetic considerations than with potential for it is not so much the magnitude of a reduction potential which is of significance as the activity or reducing power of the substance responsible for the potential. Cysteine, for instance, gives a high reduction potential but its reducing power (velocity of reduction) is greatly dependent on the presence of traces of metals which have little or no effect on the magnitude of its potential.

It is with the greatest pleasure that we express our gratitude to Sir F. G. Hopkins for the interest he has taken in this work.

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XVIII. PROTEIN METABOLISM IN CYSTINURIA.

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With regard to the anomaly of protein metabolism as seen in cystinuria, two schools of thought exist. On the one hand, Folin and his co-workers believe that there is but one type of the derangement, although it is capable of exhibiting varying degrees of severity. On the other hand, Neuberg considers that the condition is of three kinds, and that, whilst the presence of cystine in excessive quantities in the urine is characteristic of all its phases, yet it may be accompanied in some cases by tyrosine and leucine, and in others by putrescine and cadaverine. These very divergent views give the subject a complex aspect, and create, moreover, the difficulty of evolving a working hypothesis which might serve as a basis for the re-investigation of a problem not only of practical but of theoretical importance in that its elucidation would probably throw light on our present conceptions of normal protein metabolism. For these reasons further studies of individual cases appear necessary.

The present study arose from the admission to a ward of the Royal Infirmary, Edinburgh, of a patient with the following clinical history.

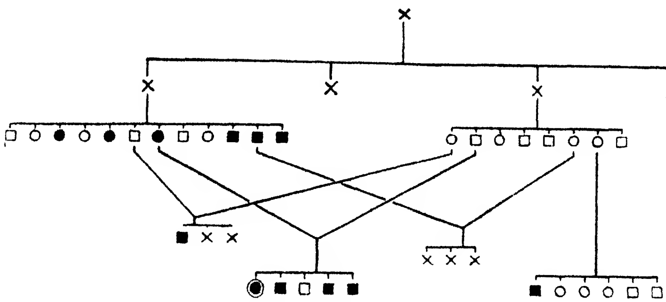
"Patient when seen was aged 23. In June, 1926, early one morning she was seized with a very acute pain in the left side of the abdomen. It was so severe that she had to get up from bed and walk about. This pain lasted for three or four hours very severely, and gradually passed off during the next two days. A good deal of vomiting was present when the pain was at its worst. Micturition caused an exacerbation of the pain. So far as she is aware she has never passed any calculi or gravel. No jaundice was present during the attack.

"Special interest was taken in the case because of the strong family history of the disease, several members having already died from the consequences of this disturbance.

"Apart from an operation for appendicitis in 1925, the patient had had no other illnesses. She was to all appearances a normal healthy girl."

Cystinuria is classified by Garrod as one of the "inborn errors of metabolism" and in this connection the reference in the above clinical notes to the occurrence of the derangement in other members of the family is highly interesting. The genealogical tree of the patient has been investigated so far as it was possible to do so, and it was found to illustrate very clearly the

hereditary aspect of the anomaly. Although there are, it is to be regretted, some gaps in the tree about which no information can be gained, nevertheless, since such records in the literature are not numerous, it is thought worthy of inclusion here (Fig. 1). The position of the subject of the present study in the family tree is indicated by the encircled figure, while crosses show the existence of members concerning whom no information is available.



□ ○ Male and female normals respectively.
■ ● Male and female cystinurics respectively.

Fig. 1.

Aim of the investigation.

The original object of the present investigation was to test by urinary analysis the dietary methods advocated by various workers in this field for the treatment of cystinurics in an endeavour to ameliorate the condition of the patient in whose family several deaths, apparently attributable, directly or indirectly, to the derangement, had occurred. While the work was in progress, however, the patient displayed such keen interest in her case that the scope of the investigation was greatly extended and additional feeding experiments carried out in order to gain further information regarding the location of the disturbance. The experiments herein described may be briefly summarised as follows.

1. Hospital diet—control.
2. Hospital diet plus sodium bicarbonate.
3. ,, ,, disodium hydrogen phosphate.
4. ,, ,, cystine.
5. ,, ,, glutamic acid.
6. Low protein diet containing a minimum amount of cystine.
7. High protein diet.

Each experiment was followed by an interval during which the patient was placed on the normal hospital diet in order to stabilise her metabolism so far as possible before the next experiment was carried out.

METHOD.

Throughout the whole of the period of investigation the protein content of the diet was carefully controlled so that the results obtained from the analyses of the urine during the different experiments might be comparable. Even under such conditions fluctuations occur and are difficult to explain. The urine was collected under toluene in 24-hour samples and on these estimations of the total nitrogen (micro-Kjeldahl), ammonia (aeration), urea (urease), inorganic, ethereal and neutral sulphur (Fiske), amino-nitrogen (Folin) and cystine (Looney) were performed. The method of estimating the cystine requires comment. In most of the recorded investigations of cystinuria the amount of urinary cystine was determined either by its actual isolation from a concentrated urine [Gaskell, 1907] or by calculation based on the assumption that the increase either in the neutral sulphur itself [Alsberg and Folin, 1905] or in the ratio of neutral sulphur to total sulphur [Mester, 1879] was due to the excretion of cystine as such. Such methods can scarcely be regarded either as being simple or accurate. For our present purpose, however, the colorimetric method devised by Looney [1922] was available, and, since preliminary attempts at the quantitative recovery of purified cystine added to both normal and cystinuric urines gave satisfactory results, the method was used throughout the investigation. While the later stages of the work were in progress a modification by Hunter and Eagles [1927] of the original method of Looney was published. The replacement of sodium carbonate by sodium hydroxide as one of the reagents in the method certainly eliminates the possibility of "cloud" formation which occasionally occurs when the original method is used. Nevertheless the results obtained by both methods on the same solutions of cystine do not differ outside the limits of colorimetric methods of estimation.

At intervals, estimations of the non-protein-nitrogen, urea and chloride were carried out on samples of blood taken from the patient.

In addition to this quantitative work, qualitative tests on the urine, sometimes concentrated under reduced pressure, for tyrosine, leucine, cadaverine and putrescine, were performed. On numerous occasions, moreover, the patient's faeces were examined for the presence of the last-named two substances.

ANALYSIS OF RESULTS.

1. *Hospital diet.* Before the main feeding experiments were attempted it was essential to discover how far the patient's metabolism could be stabilised, especially with regard to the excretion of cystine. For this purpose the patient was kept in bed and given a convalescent diet yielding on an average 1750 calories. The results obtained during the latter end of this period, given in Table I, show that the cystine output varied considerably from day to day and bore no relation to the total nitrogen in the urine. A possible reason for

Table I.

Date (1926)	Protein in diet g.	Urine cc.	Nitrogen g.			Cystine g.			Sulphur g.			Amino-N g.	Remarks
			NH_3-	Urea-	Total	Aq.	Ppt.	Total	Inorg.	Neut.	Total		
10. xi.	69	1885	0·26	4·64	9·05	0·08	0·02	0·10	1·05	0·47	1·52	0·29	
11. xi.	75	1300	0·28	4·16	8·18	0·15	0·05	0·20	0·81	0·33	1·22	0·20	
12. xi.	75	1060	0·31	3·08	6·68	0·54	0·13	0·67	0·74	0·80	1·56	0·17	
13. xi.	69	1380	0·29	2·61	7·73	0·58	0·21	0·79	0·96	0·89	1·94	0·27	
14. xi.	66	1463	0·34	2·00	7·90	0·21	0·15	0·35	0·91	0·80	1·82	0·26	

Table II.

Date (1926)	Protein in diet g.	Urine cc.	NH_3-	Urea-	Total	Aq.	Ppt.	Total	Inorg.	Neut.	Total	Amino-N g.	Remarks
6. xii.	75	1390	0·20	3·25	6·81	0·26	0·06	0·32	0·80	0·50	1·32	0·21	
7. xii.	85	1060	0·15	2·71	6·84	0·27	0·07	0·34	0·74	0·47	1·24	0·21	23·4 g. $NaHCO_3$
8. xii.	63	1830	0·23	2·34	9·15	0·56	0·09	0·65	0·96	1·06	2·04	0·27	,,
9. xii.	74	1700	0·20	3·55	8·33	0·78	0·02	0·81	1·04	0·92	1·97	0·24	,,
10. xii.	72	1400	0·20	3·58	8·54	0·38	0·15	0·53	0·99	0·65	1·66	0·22	,,
11. xii.	67	1250	0·23	3·15	7·00	0·27	0·07	0·34	0·74	0·64	1·44	0·17	
12. xii.	62	900	0·16	3·26	6·48	0·12	0·07	0·19	0·74	0·29	1·18	0·15	
13. xii.	65	800	0·26	2·94	6·36	0·23	0·05	0·28	0·65	0·34	1·13	0·16	

Table III.

Date (1926)	Protein in diet g.	Urine cc.	NH_3-	Urea-	Total	Aq.	Ppt.	Total	Inorg.	Neut.	Total	Amino-N g.	Remarks
21. xi.	60	1234	0·36	3·09	7·16	0·48	0·09	0·57	0·84	0·64	1·59	0·20	
22. xi.	67	1340	0·23	3·12	7·10	0·64	0·17	0·84	0·58	0·78	1·45	0·27	
23. xi.	78	895	0·35	3·05	6·48	0·20	0·13	0·33	0·43	0·25	0·72	0·21	6 g. Na_2HPO
24. xi.	67	1240	0·36	3·36	6·95	0·36	0·15	0·51	0·47	0·47	0·99	0·20	,,
25. xi.	72	1670	0·35	3·32	8·18	0·13	0·18	0·31	0·51	0·40	0·95	0·26	,,
26. xi.	76	1140	0·30	2·09	7·18	0·35	0·07	0·42	0·36	0·50	0·88	0·27	,,
27. xi.	72	1170	0·39	2·54	6·98	0·40	0·37	0·77	0·38	0·59	0·99	0·27	

Table IV.

Date (1926)	Protein in diet g.	Urine cc.	NH_3-	Urea-	Total	Aq.	Ppt.	Total	Inorg.	Neut.	Total	Amino-N g.	Remarks
15. xi.	66	1050	0·21	2·25	6·32	0·08	0·14	0·22	0·61	0·45	1·10	0·18	
16. xi.	65	1530	0·18	4·34	8·26	0·19	0·23	0·42	0·98	0·77	1·78	0·38	2 g. cystine
17. xi.	70	1200	0·23	3·84	8·52	0·18	0·37	0·56	1·90	0·70	2·70	0·37	4 ,,
18. xi.	74	1700	0·61	4·72	9·01	0·11	0·01	0·13	3·03	0·51	3·66	0·34	6 ,,
19. xi.	74	1000	0·54	5·00	9·40	0·26	0·02	0·28	3·74	1·26	5·02	0·16	8 ,,
20. xi.	74	1707	1·01	7·75	10·41	0·38	0·03	0·41	4·53	0·70	5·25	0·32	
21. xi.	60	1234	0·36	3·09	7·16	0·48	0·09	0·57	0·84	0·64	1·59	0·20	

Table V.

Date (1926)	Protein in diet g.	Urine cc.	NH_3-	Urea-	Total	Aq.	Ppt.	Total	Inorg.	Neut.	Total	Amino-N g.	Remarks
27. xi.	72	1170	0·39	2·54	6·98	0·40	0·37	0·77	0·38	0·59	0·99	0·27	
28. xi.	42	1210	0·25	1·80	4·92	0·50	0·11	0·61	0·23	0·22	0·47	0·17	
29. xi.	69	785	0·32	1·88	6·20	0·08	0·18	0·27	0·64	0·33	1·00	0·18	4 g. glutamic acid
30. xi.	68	1250	0·49	4·63	9·50	0·26	0·17	0·43	0·91	0·39	1·40	0·26	6 ,,
1. xii.	59	1270	0·33	4·00	9·21	0·56	0·19	0·75	0·81	0·54	1·36	0·25	8 ,,
2. xii.	70	1460	0·31	2·54	5·78	0·27	0·04	0·31	0·77	0·40	1·27	0·29	8 ,
3. xii.	64	1020	0·41	2·40	6·56	0·20	0·06	0·26	0·79	0·53	1·40	0·26	

this fluctuation was thought to lie in the varying quantity of cystine in the diet, and although it is impossible to eliminate entirely this amino-acid from the food, a dietary containing the minimum amount of cystine was fed during the remainder of the investigation. Reference will again be made to this point when the results of the experiments with the low and high protein diets (Exps. 6 and 7) are considered.

2. *Hospital diet plus sodium bicarbonate.* One of the methods advocated by different workers from time to time for the treatment of cystinurics has been the administration *per os* of sodium bicarbonate, the purpose being to render the urine strongly alkaline and so diminish, owing to the increased solubility of cystine, the liability of the formation of concrements in the urinary tract. Instances, however, are on record where such treatment even resulted in the actual reduction of the amount of cystine excreted. Thus Klemperer and Jacoby [1914] found that on feeding sodium bicarbonate the excretion of cystine gradually fell to zero only to recur when the use of this drug was stopped. More recently Looney *et al.* [1923] showed that the administration of 15–20 g. of sodium bicarbonate per day produced a definite fall in the amount of cystine excreted, and that this was accompanied by a marked disturbance in the distribution of the sulphur in the urine, indicating that the alkalosis produced had a definite influence on the course of protein metabolism.

As will be seen from Table II, which gives the results of one of several feeding experiments with sodium bicarbonate (23·4 g. per day), the findings of the latter workers could not be confirmed. The immediate effect was an increase in the amount of cystine excreted followed by a decrease to a figure a little lower than the original one. An increase in the neutral sulphur fraction took place though this is possibly related to the increased elimination of total nitrogen. It may be considered that the preliminary increase was due to the solution of cystine previously lodged in the urinary tract, but such an idea cannot be seriously entertained since similar rises occurred in three different experiments following one another at short intervals.

3. *Hospital diet plus disodium hydrogen phosphate.* The use of disodium hydrogen phosphate in preference to sodium bicarbonate for the purpose of producing an alkalosis is advocated by some workers, and an experiment was carried out on the lines of the preceding one with this compound. 6 g. were fed per day. On November 26, four days after the experiment started, the urine was definitely alkaline. At that point, however, there was a distinct fall in the amount of urea and total nitrogen excreted, a fact which is difficult to understand since the alkalinity produced by the sodium bicarbonate in the previous experiment was accompanied by a rise in the excretion of both of these components. Moreover so far as the excretion of cystine is concerned the use of disodium hydrogen phosphate offers no advantages over that of sodium bicarbonate. Results obtained during this experiment are given in Table III.

4. *Hospital diet plus cystine.* It has long been known that the cystinuric could oxidise a certain amount of ingested cystine to inorganic sulphate.

Little work of a quantitative character, however, has been done on this aspect of the problem. Moreover, with the view of attempting to find whether there was a peak in the ability of the patient to oxidise cystine given in this way, increasing amounts were given starting with 2 g. on November 16, and ending with 8 g. on November 19, so that a total of 20 g. of cystine were fed during the period of 4 days.

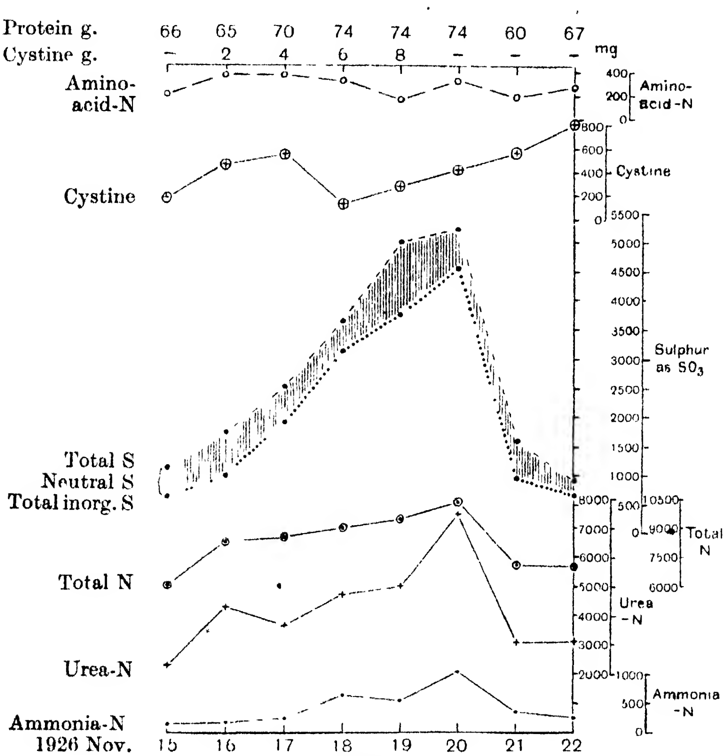


Fig. 2.

Analysis of the results obtained during the period covering this experiment (Table IV) shows that, assuming 0·60 g. (excreted on the days immediately preceding and following the period of the experiment) as the average amount of inorganic sulphate normally excreted, the additional inorganic sulphate eliminated during the feeding experiment was 11·5 g. This could only have arisen from the 20 g. of cystine fed, an amount which, theoretically, should yield 13·5 g. of inorganic sulphate. Hence some 85 % of the cystine administered must have been decomposed, a result which definitely shows that in this case at least the mechanism of the processes of oxidation of the cystine-sulphur is not at fault. Moreover, from the shape of the curve (Fig. 2) of the excretion of inorganic sulphate, it is apparent that the peak of the ability of the patient to oxidise cystine fed by the mouth had not been reached. It is to be noticed that there is no increased elimination of cystine as such during the period in question.

As will be seen from the results recorded, the increase in the inorganic sulphate in the urine is accompanied by a definite increase in the amount of ammonia excreted, a coincidence which follows from normal kidney function.

5. *Hospital diet plus glutamic acid.* One of the many peculiar features of the derangement of metabolism exhibited by cystinurics is the low ratio of urea-nitrogen to total nitrogen in the urine, and this is clearly seen from the results in the various tables to be characteristic of the patient under study. This together with the fact that there is an abnormal amount of cystine in the urine would make it appear at first sight that the processes of deamination are not functioning normally. The question then arises whether this abnormality is closely confined to the cystine molecule or is suffered generally by the whole of the amino-acids of the protein molecule. In order to study this aspect of the problem it was decided to feed a second amino-acid, glutamic, of which a small quantity was available. Following the lines adopted in the previous experiment, this was added to the diet in quantities of 4, 6, 8 and 8 g. on successive days.

From the figures recorded in Table V, it will be seen that the immediate effect was a rise in the total nitrogen and urea-nitrogen excreted, the rise in urea-nitrogen easily accounting for the additional nitrogen fed as amino-acid. It may thus be concluded that the power of general deamination is not lacking in this patient.

6 and 7. *Feeding experiments with a low protein diet* (6) *and with a high protein diet* (7). The feeding experiments with the amino-acids cystine and glutamic acid show beyond doubt that in the case under investigation the processes of oxidation and deamination are functioning normally, and that a search must be made elsewhere for an answer to the question as to what is the origin of the urinary cystine and how it escapes the normal processes of metabolism. It has already been shown (Table I) that in attempting to stabilise the patient's metabolism, it was necessary, on account of the large fluctuations in the amount of cystine in the urine from day to day, to eliminate so far as possible the cystine-rich proteins from the diet. Despite the fact that the concensus of opinion appears to favour the view that there is no connection between the diet and the amount of cystine in the urine, the results of our preliminary feeding experiments did not agree with such a conception. To gain further evidence on this point, feeding experiments were carried out in which the diet contained firstly only some 30 g. of protein and secondly 65–75 g. of the same proteins. The results of these experiments are given in Tables VI and VII respectively. That a larger amount of protein was absorbed on the high protein diet is readily seen in the higher values of the urinary total nitrogen. Accompanying this rise in the amount of protein absorbed, however, is a rise in the amount of cystine excreted from 0·71 g. (average for 9 days) to 1·1 g. (average for 9 days). This rise is large and is in almost direct ratio with the additional nitrogen eliminated, so that it is difficult to see how the conclusion that the urinary cystine is related to the protein

of the diet can be avoided. So long, therefore, as the cause of the anomaly remains unknown, it would appear that from the point of treatment cystinurics at times of crises should have their dietary protein cut down so far as is practicable, due regard being paid to the question of nitrogen equilibrium.

Table VI.

		Nitrogen					Cystine			
Date	Urine cc.	NH_3- g.	Urea- g.	Total g.	Chlorides g.	Phosphates g.	Aq. g.	Ppt. g.	Total g.	Diet
17. vi.	1050	0·159	1·395	5·25	7·5	1·198	0·495	0·105	0·600	*Low cystine*
19. vi.	1160	—	—	7·77	4·76	1·455	0·399	0·200	0·599	Protein 30 g.
20. vi.	995	0·229	3·19	5·97	3·72	1·248	0·580	0·209	0·789	Calories 1500
21. vi.	800	0·232	2·48	4·92	2·92	0·816	0·576	0·134	0·710	
22. vi.	1060	0·175	2·86	4·88	3·52	1·059	0·493	0·144	0·637	
23. vi.	1080	0·230	3·24	5·56	4·04	1·256	0·599	0·133	0·732	
24. vi.	1320	0·158	3·3	6·54	6·13	1·49	0·785	0·175	0·960	
25. vi.	1050	0·229	4·2	6·62	4·68	1·01	0·644	0·105	0·749	
26. vi.	835	0·164	2·97	5·05	3·42	1·10	0·574	0·059	0·633	

Table VII.

Date	Urine cc.	NH_3- g.	Urea- g.	Total g.	Chlorides g.	Phosphates g.	Aq. g.	Ppt. g.	Total g.	Diet
28. vi.	1150	0·245	4·26	7·02	6·77	1·44	0·573	0·114	0·687	*Convalescent*
29. vi.	1740	0·254	5·85	9·13	11·2	2·3	1·296	—	1·296	Protein 65/75 g.
30. vi.	1310	0·183	5·11	7·73	7·26	1·66	1·04	0·088	1·128	Calories 1900
1. vii.	1420	0·168	4·68	7·95	8·8	1·68	1·19	0·143	1·333	
2. vii.	1630	—	—	9·78	11·92	2·1	1·157	0·141	1·298	
3. vii.	700	0·147	3·01	4·52	4·50	1·03	0·544	0·189	0·733	
4. vii.	1625	0·205	6·5	9·75	11·9	1·82	1·38	0·182	1·562	
5. vii.	990	0·111	3·47	4·85	5·12	0·971	0·724	—	0·724	
6. vii.	1395	0·165	6·55	8·44	8·22	1·91	1·18		1·18	
7. vii.	1165	0·792	3·49	6·11	7·07	1·14	0·65	—	0·65	

Occurrence in the urine of tyrosine and leucine, and the diamines, putrescine and cadaverine. Although various workers have also reported the presence of the amino-acids, tyrosine and leucine, and the diamines, putrescine and cadaverine, yet the search for these two substances has by no means been uniformly successful. Nevertheless, the number of cases reported as showing that the excretion of abnormal quantities of cystine is only one of the manifestations of this derangement of protein metabolism was considered sufficiently large to warrant the examination of the patient's urine for the presence of these compounds. On three separate occasions during the progress of the investigation 3–5 litres of urine were concentrated under reduced pressure. In each instance the results were as follows.

(*a*) Neither leucine nor tyrosine could be isolated from the concentrate, while the colour reaction obtained with Millon's reagent was no more intense than that obtained with a similarly concentrated normal urine.

(*b*) Treatment of the concentrate with β-naphthalenesulphonic chloride according to the method of Abderhalden and Shittenhelm [1905] gave a gummy precipitate which could not be made to crystallise.

(*c*) Both the benzoylation process of Baumann and Udransky [1889] and the phenyl *iso*cyanate method of Loewy and Neuberg [1904] gave amorphous precipitates which would not crystallise after repeated attempts at purification.

It was concluded, therefore, that none of the above amino-acids or amines was present in sufficient amount to permit of the isolation of a crystalline derivative.

Blood estimations. Estimations of the blood urea-nitrogen and non-protein-nitrogen carried out at different intervals on the patient's fasting blood gave results as follows: urea-N 9·5, 10·0, 9·8 mg. per 100 cc.; N.-P.-N, 20·0, 20·5, 20·3 mg. per 100 cc. All these figures are decidedly subnormal.

Discussion.

The fact that the presence of neither the amino-acids, tyrosine and leucine, nor the diamines, putrescine and cadaverine, could be detected in the urine of the subject of the present study adds yet one more case to the growing number of cystinurics in whom the derangement of protein metabolism is limited strictly to the excretion of an abnormal quantity of cystine in the urine. Moreover, the results of the feeding experiments with the isolated amino-acid, cystine, which show that this compound is quantitatively oxidised to inorganic sulphate, confirm the findings of earlier workers in this field, *e.g.* Hele [1909] and Looney *et al.* [1923], and support the conclusion that the cystinuric oxidises cystine fed as such by the mouth in a perfectly normal manner. Such a conclusion stands in strong contradistinction to the statement of Lewis [1924] that "the failure of the organism to oxidise one specific amino-acid in cystinuria would support the theory that the processes of deamination and oxidation are not the same for all the constituent amino-acids of the protein molecule," for which little, if any, evidence exists at the present time. Moreover, the fact that the peak of the curve of the ability of the patient to oxidise cystine had not been reached, even when so much as 8 g. per day were administered, makes it difficult to understand the suggestion of Hunter and Eagles [1927] that "one of the errors of cystinuria lies in the incapacity of the liver to handle the cystine normally in reserve there." That the cystinuric can oxidise large quantities of cystine administered orally, together with the results of the final feeding experiments showing that, contrary to general opinion, the quantity of urinary cystine varies directly with the amount of protein fed, would make it appear that "the error" is not one connected either with the normal process of oxidation or with the organ in which that process takes place.

Rather would it appear that the disturbance is one closely related either to the digestive processes of the organism or to the permeability of the intestinal wall. The fact that cystine fed as such is completely oxidised while some of the cystine arising from dietary protein escapes oxidation would suggest that the amino-acid in the latter case is actually passing from the intestinal tract into the portal system, not in the isolated form, as it does normally, but as a peptide, and as such escapes the normal oxidative processes of the liver. Fission of the peptide might result when it reached the tissues

and encountered the intracellular enzymes. Cystine would then be liberated only to be excreted by the kidney owing to its presence in abnormal amount in the blood-stream.

Such an idea receives other experimental support apart from that included in this paper. Blum [1903] found that experimental cystinuria could be produced by the injection of cystine into the peripheral circulation but not when it was injected into the mesenteric venous circulation. Such facts would be met if, in the cystinuric, the passage of the liver was effected by the cystine in the form of a peptide, in which it was so linked up that it escaped the normal processes of deamination and oxidation.

A possible solution of the problem of metabolism presented by the cystinuric lies in the feeding of complex peptides containing cystine, and it is probable that a more exact knowledge of the action of enzymes on the protein molecule might give a clearer indication of the source of the urinary cystine in this type of condition.

As regards the more practical question of the treatment of cystinurics, it would appear that the only safe method is that of cutting down the dietary protein of the patient so far as is consistent with the maintenance of nitrogen equilibrium. From the results obtained there is nothing to recommend the continual use of sodium bicarbonate.

Summary.

Evidence is advanced that the cystinuric oxidises cystine to inorganic sulphate normally. Neither the amino-acids, tyrosine and leucine, nor the diamines, putrescine and cadaverine, could be found in the urine of the patient who formed the subject of the present study.

The amount of cystine in the urine appears to bear some relation to the quantity of dietary protein.

The administration of sodium bicarbonate, but not of disodium hydrogen phosphate, to the cystinuric is accompanied by a marked disturbance of the sulphur fractions of the urine. Neither of these compounds affects the excretion of cystine to any extent.

In reporting this work, the author desires to take this opportunity of expressing his deep indebtedness to Dr Robert Thin of Edinburgh for facilitating the carrying out of this research, and also for providing the exceedingly useful information regarding the genealogical tree of the subject. The work was carried out with the help of a grant from the Medical Research Council which is hereby acknowledged.

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XIX. A STUDY OF FACTORS SAID TO INFLUENCE THE NITROGEN DISTRIBUTION OF GELATIN.

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Within the past few years, several publications have appeared from the Biochemical Department of the Imperial College of Science and Technology, London, concerning the effect on the nitrogen distribution of gelatin of certain preliminary treatments of the protein. In discussing these publications and in describing experimental procedures, terminologies which have been well established by custom will be employed. By the percentage of a type of nitrogen is indicated that part of the total nitrogen of the protein which, in the hydrolysate or in a designated fraction of it, appears in this form; the basic nitrogen refers to the nitrogen of those compounds which are precipitated, under the defined conditions, by phosphotungstic acid.

According to Knaggs [1923], if gelatin is allowed, prior to the hydrolysis proper, to stand at laboratory temperature with 20 % HCl, a marked increase in the percentage of basic nitrogen takes place; the percentage of amino-nitrogen is, however, unchanged. He attributes the increase in the basic nitrogen to the formation of difficultly hydrolysable polypeptides.

Knaggs and Schryver [1924] report that while the percentage of basic nitrogen remains constant, the percentage of non-amino-nitrogen of the filtrate is increased by certain treatments of this protein. In their experiments, before hydrolysis proper, gelatin was allowed to stand at laboratory temperature with *N* HCl or *N* NaOH or was heated at 105° with water. The effect of the three treatments on the nitrogen distribution of the protein is said by the authors to be, in general, the same. In regard to the treatment with water, the percentage of non-amino-nitrogen of the filtrate is shown to increase gradually with the time of heating until it reaches a fairly constant maximum.

These two sets of results appear to be somewhat inconsistent. Moreover, the results of Thornley [1927], coming from the same laboratory, are in serious disagreement with both of them. On the authority of Thornley's experimental results and the curve that he constructed from them, the preliminary treatment of the protein with *N* HCl or *N* NaOH causes a large increase in the percentage of basic nitrogen; preliminary treatment with 20 % HCl, on the other hand, causes a smaller increase in this fraction. The former findings are contrary to

those of Knaggs and Schryver, the latter are contrary to those of Knaggs. In agreement with Knaggs and Schryver, however, Thornley finds that heating gelatin with water causes practically no change in the basic nitrogen fraction. He finds, further, that subsequent treatment of the same material with HCl does not cause an increase of this percentage. Figures for non-amino-nitrogen are not included in his published results.

Finally, Schryver and Buston [1927] attribute the increase in the percentage of basic nitrogen, which they observed, to *dl*-lysine. The details of their isolation of this amino-acid are reported.

In many cases, we are unable to deduce from these publications the number of duplicates which have contributed to the results and the degree of agreement of these duplicates. There are, in addition, various inconsistencies in the conclusions drawn from the experimental work. The investigators agree, however, that the nitrogen distribution of gelatin is altered by preliminary treatment of the protein with acid or alkali. Were this fully established, it would be necessary to conclude that the protein molecule is much more labile than has been hitherto supposed. The figures appearing in the literature as nitrogen distribution numbers could not, moreover, be regarded as definite for the proteins in question. In view of the importance of these data, Professor Sørensen suggested to the writer that an investigation of the subject be carried out. The results obtained by this investigation show no change in nitrogen distribution due to preliminary treatment of gelatin with acid or alkali, and fail, accordingly, to support the principal contention of the investigators mentioned.

Experimental.

In order to become thoroughly familiar with the methods of analysis and to investigate certain parts of the procedure, we carried out twenty preliminary nitrogen distribution experiments. The samples of gelatin used for these analyses were divided into groups on the basis of the treatment given them prior to the hydrolysis proper. Of the results, only those which pointed the way to the experimental method adopted are mentioned. The nitrogen distribution numbers recorded in Table IV under their group headings (A, B, C and D) are derived from twelve later analyses of gelatin. The details of the method used for obtaining and classifying them are given below. The conclusions to be drawn from the two sets of experiments are the same but the earlier ones were carried out on smaller amounts of material.

Treatment and hydrolysis of gelatin.

The different treatments with acid and alkali accorded to gelatin by Knaggs and by Schryver were duplicated as closely as was compatible with obtaining uniformity of samples. To obtain the desired uniformity, the protein was dissolved in hot water, care being taken to obtain a homogeneous solution. The ratio by weight of gelatin to water was approximately 11 : 100. 100 cc.

portions of the hot solution were pipetted into flasks and weighed. Upon the cooling of the material, gels formed.

The total nitrogen per gram of solution was determined on a separate sample. 20 cc. were diluted to a volume of 500 cc., and portions of the dilute solution were analysed. Here, and wherever possible in subsequent steps of the procedure, solutions and the parts of them used for analysis were weighed. Where this was not feasible, a method of calibration was employed.

The preliminary treatments of the 100 cc. samples are described in the following paragraphs. The concentration of HCl in each of the solutions was brought to 20 % before the hydrolysis proper of the gelatin, which was accomplished by boiling the acid solution for 24 hours. Hang-in reflux condensers were used.

Group A. No preliminary treatment. The gel was liquefied by heat and the resulting solution heated almost to boiling. A hot solution of HCl was added and the gelatin immediately hydrolysed.

Group B. Treatment with N *NaOH.* The gel was liquefied by heat and a solution, containing the calculated amount of NaOH, was added; the flask was thoroughly shaken and at once plunged into a cooling-bath. The mixture was allowed to stand for 24 hours at room temperature. At the expiration of this time, a hot solution of HCl was added and the gelatin was at once hydrolysed.

Group C. Treatment with N *HCl.* The gel was liquefied by heat and a solution containing the calculated amount of HCl was added. The procedure given for Group B was then followed except that the time of standing was 48 hours.

Group D. Treatment with 20 % *HCl.* The gel was liquefied by heat, a solution of HCl of proper strength was added and the flask was plunged into a cooling-bath. As soon as the mixture had stood for 24 hours at room temperature, the gelatin was hydrolysed.

Group A, then, consisted of controls, approximately 10 g. of protein being hydrolysed by some 25 times its weight of 20 % HCl. The members of Group B differed in that, prior to the hydrolysis proper, they were allowed to stand for 24 hours with *N* NaOH and in that the hydrolysis was accomplished in the presence of NaCl. The members of Group C differed from the controls in that they were allowed to stand for 48 hours with *N* HCl, and the members of Group D in that they were allowed to stand for 24 hours with the acid used for hydrolysis.

Comparison with the publications of Knaggs and of Knaggs and Schryver will show the similarity of the experimental conditions. The chief point of difference is that, in our experiments, the gelatin was first heated for a short time with water. According to Knaggs and Schryver, any noteworthy change in the protein, due to this treatment with water, would make itself apparent by an increase in the percentage of the non-amino-nitrogen of the filtrate. It is extremely unlikely that such an increase would be compensated by

a corresponding decrease in the non-amino-nitrogen of the precipitate, particularly since the total nitrogen of the latter fraction was reported by Knaggs and Schryver as remaining unchanged. A change in the protein should, consequently, be accompanied by an increase in the percentage of total non-amino-nitrogen.

To make certain that such a change had not occurred, a control determination was carried out. Gelatin was dropped into boiling HCl and hydrolysed and the ammonia and humin were removed from the hydrolysate. Portions of the residue were used for determinations of total nitrogen and of amino-nitrogen. By the formaldehyde titration, 17·81 % and by the nitrous acid method, 27·80 % of the total nitrogen of the hydrolysate was in the non-amino-form in this residue. Corresponding figures for the members of Group A average 17·82 % and 26·97 % respectively. From the non-amino-nitrogen test of two of the above investigators, then, it would seem that our treatment with water did not greatly alter the protein material. Our preliminary treatments of gelatin should, consequently, be justly comparable to theirs.

To make sure that no errors would arise from incomplete hydrolysis of the protein, the method adopted was compared with hydrolysis for 1½ hours in the autoclave at approximately 150°. Samples hydrolysed in the autoclave gave no greater figures for amino-nitrogen but higher figures for ammonia-nitrogen than those hydrolysed at ordinary pressures. Hydrolysis at ordinary pressures for 24 hours was, therefore, deemed sufficient. Since the figures for ammonia-nitrogen obtained by these experiments are needed for a part of the discussion in the next section, they are given in Table I.

Table I.

Hydrolysis	% of total N as ammonia-N
Ordinary pressure: 20 % HCl	1·49 (average of 8 determinations)
Autoclave: 10 % HCl	2·27 (average of 6 determinations)
Autoclave: 20 % HCl	2·81 (average of 6 determinations)

Maximum deviation of an individual figure from any average: 0·36 % of total nitrogen.

Determination of ammonia-nitrogen.

For the removal of ammonia, MgO and boiling at ordinary pressures, CaO and boiling at low pressures, and NaOH and aeration have been used by different investigators. The first and second of these methods, however, have been severely criticised. As noted by Jodidi and Moulton [1919], MgO, in excess of that required to release the ammonia and saturate the solution, occludes some nitrogen-containing substances other than those correctly given the name of humin. The amount of nitrogen occluded is roughly proportional to the amount of excess MgO and the substances are not removed by washing the oxide with hot water. Our results confirm this observation. The larger the amounts of MgO used, the higher are the apparent values for humin-nitrogen. Although it has not been tested, it is likely that CaO as well causes

an error at this point. Treatment of the excess oxide with HCl before filtering should, by giving soluble products, eliminate this error.

The MgO and CaO procedures, as originally presented, must be criticised on another count. As will appear in the section on precipitation with phosphotungstic acid, the amount of inorganic salt introduced into the solution of amino-acids should be known. The original oxide methods are unsatisfactory in this respect. Thimann [1926] gives a thorough discussion of this point. Although his conclusions are to the contrary, it seems to us that this offers a second important reason for treating the excess oxide with HCl. If a known amount of oxide is added to the hydrolysate and this oxide is completely changed to the soluble chloride, it is self-evident that the amount of salt introduced into the solution can quickly be calculated.

Owing to the fact that cystine is not present in the hydrolysate from gelatin, the question of its decomposition by heating to 100° in alkaline solution need not be considered. Either of the oxide methods, altered as indicated, should be adequate to the purpose at hand. For this particular investigation, however, it was not judged advisable to make use of either of them. NaCl was earlier introduced into the members of Group B by the preliminary treatment of the protein samples with NaOH and the subsequent acidification. Complication of matters by the introduction of a second salt was not desired. This consideration limited us to a procedure involving the use of NaOH.

In the MgO method, the hydrolysate is heated to 100° in alkaline solution. Such a practice should be as permissible in any other method, provided that the p_H of the solution be no higher, a factor which may be controlled by the use of the proper indicator. The procedure finally adopted entails the use of NaOH and boiling at ordinary pressures, the p_H being controlled by titration of the solution with phenolphthalein as the indicator. Before its adoption, it was compared to the MgO method. The results of this comparison are given in Table II. From them, it may be seen that the amounts of ammonia obtained by the two methods do not greatly differ and that the addition of several millimols of NaOH in excess of the proper amount does not markedly affect the results.

Table II.

MgO (g.)	% of total N as ammonia-N (average of duplicates)
3	1·32
4	1·35
1·6 *N* NaOH (cc.)	
54	1·32
55	1·34
56	1·36
57	1·36
60	1·39
65	1·40

Maximum deviation from any average: 0·02 % of total nitrogen.

The method was applied as follows. A hydrolysate, after being twice concentrated to small volume on the steam-bath to remove HCl, was introduced into a 1·5 litre flat-bottomed flask and a few drops of phenolphthalein were added. The solution was then titrated with NaOH to a distinct pink. Additional NaOH, sufficient in amount to react with the ammonium chloride present, was added and the ammonia boiled off. Immediately after the removal of the ammonia, the solution was acidified with HCl, a procedure designed to stop any decomposition of amino-acids. It is unlikely, however, that with either this or the MgO method there is a complete absence of amino-acid decomposition. In a series of eight determinations with MgO, water was added to the residue from the usual distillation and a second distillate was collected and analysed. The ammonia-nitrogen, obtained from the second distillation, averaged 0·07 % of the total nitrogen of the protein. Although these results imply that the treatment is too drastic, the error indicated is small when compared to the total error of the determination.

As may be seen from Tables I and IV, the figures obtained for the ammonia-nitrogen of analogous solutions vary greatly. On the other hand, duplicate analyses of a single solution, as given in Table II, show no such variation. From Table I, it may further be seen that different conditions of hydrolysis give rise to ammonia-nitrogen values of different magnitude. This was remarked for a number of proteins by Henderson [1899] and for gelatin by Denis [1910]. These observations, taken together, suggest that a lack of constancy of the ammonia-nitrogen figures in duplicate nitrogen distribution experiments is due to differences at present uncontrollable in the conditions of hydrolysis of the protein samples.

Determination of humin-nitrogen.

The residue, after the distillation of ammonia and the subsequent acidification, was filtered into a 250 cc. volumetric flask and the filter-paper was thoroughly washed. The precipitate was analysed and the result recorded as humin-nitrogen.

The filtrate was made up to a volume of 250 cc. and parts of it were used for determinations of amino-nitrogen and of total nitrogen and for the precipitation of the bases with phosphotungstic acid; one 5 cc. portion was titrated to neutrality. The figure obtained by this titration is of value in adjusting any sample to a definite acid concentration. This adjustment is necessary before the decolorisation for the formaldehyde titration and in the preparation of the solution for the precipitation of the bases.

Determination of amino-nitrogen[1].

By the nitrous acid method. This was carried out on 5 cc. samples according to the method of Van Slyke [1912], except that the sample was introduced

[1] When this investigation was carried out (spring 1927), the method of Linderstrøm-Lang [1927, 1928] was not elaborated.

by means of a calibrated pipette as recommended by Plimmer [1924]. The temperature was always 20° or more, so a reaction time of 30 minutes was allowed.

By the formaldehyde titration. This was carried out according to the directions of Jessen-Hansen [1923], with a modification, used in the Carlsberg Laboratory and involving the substitution of bromothymol blue for litmus paper as the indicator in the neutralisation. The use of separate samples for neutralisation and for titration with formaldehyde is thus necessitated.

A 70 cc. portion of the solution of amino-acids was used for the decolorisation and, of the resulting 100 cc., 40 cc. were used for the treatment to remove CO_2. Of the 50 cc. of CO_2-free filtrate, two 20 cc. portions were used for the analysis itself.

Precipitation with phosphotungstic acid.

Thimann [1926] reported that the precipitation of the bases with phosphotungstic acid is influenced by the concentrations of salts and of the mineral acid. Our results confirm these observations but were obtained under somewhat different experimental conditions and are, consequently, of a different magnitude. The results of these control experiments are given in Table III.

Table III.

Approximate molarity in			N (mg.) in precipitate (average of duplicates)
NaCl	$MgCl_2$	HCl	
0·01	—	1·2	13·6
0·44	—	1·2	15·2
0·87	—	1·2	16·5
0·01	0·25	1·2	14·7
0·01	0·50	1·2	15·5
0·01	—	2·3	14·4

Total nitrogen of each sample: 87·2 mg.

For the basic figures of two series of nitrogen distribution numbers to be strictly comparable to each other, then, the concentrations of acid and of salts in the solutions used for precipitation with phosphotungstic acid must be the same. The concentration of acid may easily be controlled by the determination of the acidity of each solution and by the addition of an amount of acid sufficient to bring the total concentration to the required figure. Control of the salt concentration may be accomplished by the use for hydrolysis of protein samples of equal or of known salt content, by ascertaining the amounts of salt introduced into the solution at any point in the procedure, and by a final adjustment of each solution to a definite salt concentration. The magnitude of the fluctuations in the basic fraction to be expected from a failure to control the concentrations of acid and of salt may be calculated from the figures in Table III. For this investigation, they would have been perceptible. The concentrations of NaCl in the 200 cc. precipitating solutions, arising from the NaOH used for boiling off the ammonia, varied from 0·33 N to 0·38 N. The acidity of the solutions varied from 0·07 N to 0·14 N.

The concentration of nitrogen, also, in each of the solutions used for precipitation of the bases should be kept at a constant value. This is amply demonstrated by the work of Kernot and Knaggs [1928], which is well supported by theoretical considerations. Van Slyke [1911] recognised that the phosphotungstates of the bases have solubilities great enough to be of importance. These solubilities have not been determined in the presence of known amounts of salts.

Lastly, the solution in which the precipitation of phosphotungstates is taking place should be kept at a definite temperature and a definite time should be allowed for the precipitation. A variation in the solubility of phosphotungstates with temperature was reported by Gortner and Hoffman [1925] and this variation was confirmed by Kernot and Knaggs [1928]. Our results, also, afford a confirmation. Two filtrates from 48-hour precipitations at 18° were allowed to stand an additional 48 hours at 4°. Additional precipitates, the nitrogen of which averaged 2·45 % of the total nitrogen of the protein, were obtained. Plimmer and Rosedale [1925] reported that the percentage of basic nitrogen is influenced by the time allowed for the precipitation to take place.

For our experiments, the volume in which each precipitation was allowed to take place was 200 cc., the concentrations of HCl and NaCl were 1·0 N and 0·75 N respectively; and the nitrogen in the solution before precipitation was 900–908 mg. Forty-eight hours were allowed for each precipitation to take place and the temperature of the solutions during this period was 18°.

In preparation for this precipitation, 150 cc. of the solution of amino-acids were introduced into a 300 cc. Erlenmeyer flask and the calculated amounts of HCl, NaCl and water were added. The solution, the volume of which at this point was 190 cc., was now heated almost to boiling and 15 g. of phosphotungstic acid in 10 cc. of solution were added. The flask was then allowed to stand for 48 hours in a thermostat at 18°. The precipitate of phosphotungstates appeared only after the solution began to cool. If the precipitate was allowed to form in the cold, it was found almost impossible to redissolve it by heating. Further, its manipulation was difficult and the results were erratic.

Filtration and washing of precipitate. The precipitate was collected and washed according to the method of Van Slyke [1915], except that the amount of solution used for washing was carefully controlled, as recommended by Plimmer and Rosedale [1925]. For the amount of precipitate with which we were dealing, five 20 cc. portions of the wash liquid recommended by Van Slyke, cooled to about 0°, appeared to be satisfactory, and this amount was accordingly adopted as a standard.

Treatment of the precipitate. The precipitate was dissolved in approximately N NaOH as directed by Plimmer and Rosedale. Of the resulting 100 cc., 10 or 20 cc. portions were used for the determination of total nitrogen. The remainder was used for the determination of amino-nitrogen by one of the methods mentioned above. For the nitrous acid method, 10 cc. samples were

used. For formaldehyde titrations it was necessary to remove the phosphotungstic acid and to make sure that the solution was free from CO_2. From 60 to 80 cc. the phosphotungstic acid was removed by the $BaCl_2$ method of Van Slyke [1911], after which the solution was acidified and evaporated to a small volume on the steam-bath. It was then given the treatment outlined by Jessen-Hansen for the removal of CO_2, by which the last traces of phosphotungstic acid also were removed. Finally, the solution was filtered into a 50 cc. volumetric flask and two 20 cc. portions were used for the analysis.

Treatment of the filtrate. The filtrate and washings were transferred to a 500 cc. volumetric flask and diluted to volume. 20 cc. samples were used for the determination of total nitrogen. From 250 cc. phosphotungstic acid and CO_2 were removed in the same manner as above and the solution of amino-acids was filtered into a 100 cc. volumetric flask. Of this, two 20 cc. portions were used for the formaldehyde titration and 5 cc. samples were used for the nitrous acid treatment.

Discussion of results.

The results of these experiments are given in Table IV. From them, it can hardly be concluded that preliminary treatment of gelatin with acid or alkali affects the nitrogen distribution. The possible exception is that the increase in the percentage of ammonia-nitrogen observable in the members of Group B may be due to the preliminary treatment of the protein with alkali. That this may not be the correct interpretation, however, is indicated by the statements of Hart [1901] and Kossel and Patten [1903] that the percentage of ammonia-nitrogen is increased by the addition of NaCl to the hydrolysing acid.

As has been stated, the solutions and the parts of them used for the various analyses were, whenever possible, weighed. Since no attempt was made to use exactly aliquot portions, it might be misleading to record the actual values of the determinations. The approximate magnitude of each analysis may easily be calculated from the figures for total nitrogen and the records of volume scattered throughout the discussion.

It is worthy of note that the values for total amino-nitrogen obtained by direct analysis are considerably higher than those obtained by adding the values for the amino-nitrogen of the precipitate to the figures for the amino-nitrogen of the filtrate. In the case of the nitrous acid method it is not difficult to assign the reason for this anomaly. Van Slyke [1911] advocates a reaction time of 10 minutes when lysine is absent from the solution to be tested and a reaction time of 30 minutes when lysine is present. This advice was followed. The filtrate from the bases was thus allowed a reaction time of 10 minutes while the other solutions were each allowed 30 minutes. In preliminary experiments it was found that four filtrates, which gave an average of 60·9 % amino-nitrogen for 10 minutes' reaction, gave an average of 65·8 %

Table IV.

A comparison of the nitrogen distribution numbers of treated and untreated gelatin.

Group	Total N found mg.	NH_3-N found (a) %	Humin-N found (b) %	Total amino-N found: Form. titration %	Total amino-N found: HNO_2 method %	Total non-amino-N calculated: Form. titration %	Total non-amino-N calculated: HNO_2 method %	Total amino-acid-N found (c) %	Total N (a)+(b)+(c) %
A	1529	1·60	0·06	80·57	71·45	18·11	27·23	98·68	100·34
A	1532	1·63	0·05	80·34	71·71	18·15	26·78	98·49	100·17
A	1532	1·39	0·04	81·45	71·75	17·22	26·92	98·67	100·10
B	1529	1·83	0·06	80·79	72·55	17·82	26·06	98·61	100·50
B	1532	1·68	0·05	80·92	71·18	17·52	27·26	98·44	100·17
B	1532	1·65	0·05	81·19	71·24	16·79	26·74	97·98	99·68
C	1530	1·58	0·05	80·05	71·45	18·50	27·10	98·55	100·18
C	1530	1·62	0·06	80·50	72·16	17·96	26·30	98·46	100·13
C	1531	1·52	0·04	80·92	71·84	17·71	26·79	98·63	100·19
D	1530	1·54	0·04	80·74	71·17	17·89	27·46	98·63	100·21
D	1530	1·78	0·07	79·98	71·33	18·19	26·84	98·17	100·02
D	1532	1·31	0·05	80·88	71·95	17·75	26·98	98·63	99·99
Av. A	—	1·54	0·05	80·79	71·64	17·82	26·97	98·61	100·20
Av. B	—	1·72	0·05	80·97	71·66	17·37	26·68	98·34	100·11
Av. C	—	1·57	0·05	80·49	71·82	18·06	26·73	98·55	100·17
Av. D	—	1·54	0·05	80·53	71·48	17·95	27·00	98·48	100·07
Av. of all	—	1·59	0·05	80·70	71·65	17·80	26·85	98·50	100·14

Amino-N of filtrate found: Form. titration %	Amino-N of filtrate found: HNO_2 method %	Non-amino-N of filtrate calculated: Form. titration %	Non-amino-N of filtrate calculated: HNO_2 method %	Total N of filtrate found (d) %	Amino-N of precipitate found: Form. titration %	Amino-N of precipitate found: HNO_2 method %	Non-amino-N of precipitate calculated: Form. titration %	Non-amino-N of precipitate calculated: HNO_2 method %	Total N of precipitate found (e) %	Total N (a)+(b)+(d)+(e) %
69·52	58·46	12·26	23·32	81·78	—	8·00	—	8·66	16·66	100·10
69·85	58·76	12·37	23·46	82·22	8·07	—	8·45	—	16·52	100·42
70·99	59·34	11·26	22·91	82·25	—	—	—	—	16·14	99·82
68·63	57·87	13·12	23·88	81·75	—	7·74	—	8·75	16·49	100·13
70·53	58·80	10·80	22·53	81·33	7·93	—	8·73	—	16·66	99·72
71·49	59·73	10·32	22·08	81·81	—	—	—	—	16·43	99·94
70·11	57·93	11·31	23·49	81·42	—	7·89	—	8·98	16·87	99·92
69·11	58·03	12·54	23·62	81·65	7·96	—	8·59	—	16·55	99·87
70·61	58·34	11·38	23·65	81·99	—	—	—	—	16·22	99·77
70·62	58·65	11·44	23·41	82·06	—	7·95	—	8·40	16·35	99·99
69·35	58·06	12·44	23·73	81·79	7·87	—	8·54	—	16·41	100·05
70·68	59·15	11·20	22·73	81·88	—	—	—	—	16·52	99·76
70·12	58·85	11·96	23·23	82·08	—	—	—	—	16·44	100·11
70·22	58·80	11·41	22·83	81·63	—	—	—	—	16·53	99·93
69·94	58·10	11·75	23·59	81·69	—	—	—	—	16·55	99·86
70·22	58·62	11·69	23·29	81·91	—	—	—	—	16·43	99·93
70·13	58·59	11·70	23·24	81·83	—	—	—	—	16·49	99·96

amino-nitrogen for 30 minutes' reaction. This accounts for the greater part of the discrepancy. The temperature at which these reactions were carried out varied from 20° to 23°.

An attempt was made to discover the cause of the loss of formaldehyde-titratable nitrogen. Since there was no loss in total nitrogen, this must have occurred in preparing the solutions for the titration. In experiments carried through for this purpose, all precipitates arising from the addition of $BaCl_2$ to the solutions containing phosphotungstic acid were analysed for nitrogen. Despite a thorough washing of these precipitates, the analyses invariably showed the presence of considerable and nearly constant amounts of nitrogen. The amounts were higher in the precipitates from the mono-amino-fraction than

in those from the basic fraction, probably owing to the larger part of the phosphotungstic acid being associated with the first fraction. To the solutions from which the phosphotungstic acid had been removed, additional amounts of this acid were added and the $BaCl_2$ treatment was repeated. Additional amounts of nitrogen were removed from the solutions by this treatment. When the precipitation of 7·5 g. of phosphotungstic acid was carried out in a volume of approximately 800 cc. an amount of nitrogen, roughly corresponding to 1·5 % of the nitrogen present in the solution, was removed. This held true whether the solution in question was one of basic amino-acids or one of mono-amino-acids. Although insufficient data have been accumulated to permit of any definite statement, the indications are that a considerable loss of nitrogen is unavoidable when phosphotungstic acid is precipitated from a solution of amino-acids by means of $BaCl_2$ and that this phenomenon is not a selective one but depends on the concentrations of the amino-acids and of the phosphotungstic acid in the solution.

For future work, the more recent amyl alcohol-ether method of Van Slyke [1915] will be used for the removal of phosphotungstic acid. From test experiments carried out on gelatin, the separation of the amino-acids from phosphotungstic acid by this method seems to be very nearly quantitative. A small amount of the latter acid remains with the amino-acids and is precipitated upon treatment of the solution with $BaCl_2$ for the removal of CO_2. However, the amount is so small that less than 0·1 % of the total nitrogen is removed at this point.

Summary.

1. The experimental results force us to the conclusion that, within the limits of accuracy of the method described, preliminary treatment of gelatin with acid or alkali has no effect on the percentages of non-amino-nitrogen and of basic nitrogen.

2. Several other factors have been reported by various workers as influencing the figures obtained as nitrogen distribution numbers of proteins. A study of several of these factors has been made. From the results of this study, certain modifications of the method of analysis of proteins are advocated. It is believed that the numbers obtained by the modified method will be, within certain limits of experimental error, fairly comparable to each other. The following paragraph gives a brief summary of the procedure as altered, particular stress being laid on the alterations.

The protein is hydrolysed by boiling with 20 % HCl. After removal of as much of the HCl as is possible by evaporation, the hydrolysate is rendered alkaline by the addition of a known amount of MgO, CaO or NaOH and the ammonia is removed under the proper conditions. The residue is acidified and the humin is filtered off. The filtrate, of which the acidity is determined, is divided into aliquot portions; some of these are used for determinations of amino-nitrogen and some for total nitrogen. In one portion the amino-acids

are divided into two groups by a precipitation with phosphotungstic acid. The concentrations of salt or of salts, of acid and of nitrogen are always brought to definite values in this latter portion before the precipitation is allowed to take place. The solution of amino-acids is heated almost to boiling before the phosphotungstic acid is added, and the resulting mixture is kept at a definite temperature during the definite time allowed for precipitation. The phosphotungstates are filtered off and are washed according to a rigidly defined technique. If determinations of the amino-nitrogen of the filtrate and of the precipitate are to be made by the nitrous acid method only, no preparation of the filtrate is necessary and the precipitate may be obtained in a suitable form by its solution in N NaOH. Should it be desirable to make use of the formaldehyde or alcohol titrations, the phosphotungstic acid is removed from each of the amino-acid fractions by the amyl alcohol-ether method. The amino-nitrogen and the total nitrogen of the filtrate and of the precipitate are determined.

In conclusion, I wish to thank Professor S. P. L. Sørensen for the suggestion of this problem and for his kind and helpful criticism and advice during the execution of the work. To Cheney Bros. of South Manchester, Conn., I am indebted for the Fellowship which permitted the research to be undertaken.

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XX. THE AMINO-ACIDS OF FLESH.

III. THE DIAMINO-ACID CONTENT OF FISH.

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Previous analyses [Rosedale, 1922, 1928] led to the broad conclusion that normal mammalian tissues showed but little variation in their diamino-acid composition. The methods employed, however, seemed sufficiently sensitive to detect certain differences in some pathological tissues. The work reported in this paper was undertaken as an extension of the study of food proteins and deals with types of fish which constitute a very important source of food in this part of the world. In some districts, owing to situation as well as to religious sentiment, fish forms almost the only source of protein. While the prowess of the Malay as a sea-fisherman is well known, the development of the culture of carp is a local industry which merits attention, not only from the point of view of increasing the food supply, but also on account of the fact that ponds which can be made to rear these fish successfully cease to be suitable breeding grounds for mosquitoes. Practically the whole of a fish is used as human food. Heads, skins, etc. are offered for sale in local markets and form a cheap commodity used by the poorer masses of the population. It has not been possible to analyse every species of fish which finds its way into Singapore markets, but a selection has been made of those which are most commonly found, and which serve as general types.

While slight differences may be noted between these tropical fish and mammalian tissue which was analysed in Europe, the results cannot, on the whole, be said to be very divergent. In the following list of the specimens which have been included in this work the local name is given in brackets: red snapper, *Lutianus roseus* (Ikan merah); horse mackerel, *Caranx rottleri* (Chencharu); tunny, *Thynnus* (Tongkol); thread fins, (1) *Polynemus indicus* (Kurau), and (2) *P. tetradactylus* (Senangin); carp, *Thynnicthys* (two species locally called Twatow and Song Hu respectively). The local names are taken for the most part from Maxwell [1921]. In addition to the muscle meat of the above, analyses have been made of the roes and liver of sting-ray (Pari beting), the skins of horse mackerel, and the heads of carp (Twatow). The horse mackerel and Kurau were specimens of dried fish, and the horse mackerel and tunny are red-fleshed varieties.

Experimental.

The preparation of the muscle tissues has followed exactly the procedure of Rosedale [1928], which consists in extraction with 0·1 % acetic acid and subsequent digestion with pepsin. The livers, skins and heads were ground, dried and thoroughly extracted with ether prior to carrying out the above treatment. The preparation of the roes is dealt with in another section. After digestion with pepsin, the protein was hydrolysed by 25 % HCl and analyses were carried out by Van Slyke's method as described by Plimmer and Rosedale [1925] and in certain cases also by a modification of the method of Kossel. The analyses by Van Slyke's method have been made at least in quadruplicate.

Irregularities of the Van Slyke method.

The several analyses of tunny and of the dried fishes were not so concordant as were those of the fresh white-fleshed varieties and compared less favourably in this respect with the former analyses of mammalian tissues. Kernot and Knaggs [1928] consider these discrepancies to be due to the temperature and mode of precipitating with phosphotungstic acid, and give directions towards improvement of this technique. Since however the temperature of this laboratory is higher than is usual in Europe, and since concordant results had been secured with fresh white-fleshed fish without resort to the precautions of these authors, it was felt that the cause of error must be sought elsewhere. It was decided to make a study of the hydrolysis by acid.

Table I. *Results of variation in time of hydrolysis.*

Percentage of total nitrogen.

				Diamino-fraction		Monoamino-fraction		
	Time (hours)	Amide-N	Humin-N	Total N	Amino-N	Total N	Amino N	% of N
Tunny	5	5	1	36	20	54	51	96
	10	8	1	39	20	55	54	103
	15	8	1	30	21	63	58	102
	40	9	1	27	20	62	60	99
	60	9	1	27	21	63	62	100
	80	9	1	26	20	67	61	103
	100	9	1	26	20	67	61	103
Carp (Twatow)	10	6	1	46	20	59	57	112
	25	7	1	33	16	62	58	103
	40	7	1	27	17	63	61	98
	100	7	1	27	18	63	62	98
Kurau	25	6	1	35	17	61	54	103
	40	6	2	31	21	61	54	100
	60	6	1	26	18	62	54	95
	100	8	2	27	18	63	54	100
Horse mackerel (flesh)	36	4	1	39	11	41	37	85
	60	6	1	27	15	59	51	93
	100	8	1	28	16	62	56	99
Horse mackerel (skins)	36	6	11	27	22	52	48	96
	100	6	12	20	11	64	61	102

Experiments were made with different varieties of tissue, in which the only alteration in the Van Slyke procedure was a variation in the time of hydrolysis with 25 % HCl. A duplicate analysis was made in each experiment, and the results are given to the nearest whole number in Table I.

These figures show that, when hydrolysis is incomplete, material which cannot correctly be considered as diamino-acids is precipitated by phosphotungstic acid. As hydrolysis proceeds, the proportion of the monoamino-acid fraction becomes greater. In the case of the fresh fishes, tunny and carp (Twatow), hydrolysis appears to be complete by the 80th and 40th hour respectively.

Experiments with Kossel's method.

In a former paper [Rosedale, 1928] a comparison was made between the Van Slyke and Kossel methods. No fundamental differences appeared in the results other than those due to losses sustained in the manipulation, and it seems evident that the Van Slyke method is capable of giving as accurate information of the diamino-acid composition of proteins as any available method. Vickery and Leavenworth [1928] published a modification of Kossel's method in which the separation of histidine from arginine is brought about by the exact neutralisation of the mixture, at which point the histidine is completely precipitated as the silver salt, arginine remaining in solution until it is made alkaline. This procedure differs from that of Rosedale [1928], who precipitates histidine from acid (1·5 %) solution. An experiment was therefore carried out on the flesh of Senangin in two portions, one portion being treated according to the directions of Vickery and Leavenworth, and the other according to the writer's modification [1928].

Table II. *Results comparing the two modifications of Kossel's method.*

Total nitrogen of each portion = 7·224 g.

	V. and L. method	R. method
Total N of diamino-ppt.	—	2·1 g. = 29 %
,, histidine portion	0·458 g. = 6·3 %	0·301 g. = 4·1 %
,, arginine portion	0·612 g. = 8·4 %	0·717 g. = 9·9 %
,, lysine portion	0·419 g. = 5·7 %	0·421 g. = 5·8 %

The histidine portion of the Vickery and Leavenworth method was made up to 1 litre and arginine estimations according to the directions of Plimmer [1916] were carried out upon 20 cc. portions. These gave an average value of 0·0981 g. arginine-nitrogen, which corresponds to 21 % of this fraction. Plimmer [1916] states that in using glass flasks, as was done in this case, the extent of the decomposition of histidine is from 1·5 to 3 % of its nitrogen. Consequently, in spite of great care used in adjusting the bulky solution to p_H 7·0 before precipitation of the histidine, it appears that some arginine was carried down with the silver salt of histidine and that it was not excluded by the subsequent reprecipitation with mercuric sulphate. A few of the other tissues have been analysed by Kossel's method and the results are given in Table III.

Table III. *Results by Kossel's method.*

Percentage of total N.

	Senangin	Horse mackerel	Carp heads (Twatow)
Histidine-N	4	3	1
Arginine-N	10	9	11
Lysine-N	6	8	5
No. of exps.	3	2	2

Comparison with the Van Slyke results (Table IV) shows that these figures are as usual lower than the Van Slyke figures.

Experiments with roes.

Male and female roes were worked up separately. They were treated in accordance with the directions of Kossel and Dakin [1904] for the preparation of protamine sulphate, by precipitating the minced tissue with acetic acid, boiling the precipitate, and reprecipitating with alcohol after treatment with dilute H_2SO_4. A large precipitate was thus obtained, but in the case of the female roes much of the precipitate redissolved on standing at room temperature. The remaining portion of this precipitate was filtered off, and formed fraction I (see Table IV). An attempt to reprecipitate the filtrate by the use of more dilute H_2SO_4 and alcohol produced a small quantity of material which formed fraction II (Table IV). In the case of the male roes, the first precipitate was filtered off without being allowed to stand. This forms fraction I of the male roes (Table IV). The residual tissues of both male and female roes were coagulated in the same way as the ordinary flesh tissues, forming fraction II of the male and fraction III of the female roes (Table IV). Digestion with pepsin was followed in every case by hydrolysis with 25 % HCl and the usual Van Slyke procedure. The general results by Van Slyke's method are presented in Table IV.

Table IV. *Results by Van Slyke's method.*

Percentage of total N.

	Time of hydrolysis (hours)	Amide-N	Humin-N	Diamino-fraction					Monoamino-fraction		Total
				Total N	Amino-N	Arginine-N	Histidine-N	Lysine-N	Total N	Amino-N	
Male roes, fraction I	40	15	9	49	14·5	41	6	1	31	29	104
,, II	40	14	1	24	15	12	0	12	58	57	97
Female roes, fraction I	40	15	6	48	27·5	37	7	4	32	31	101
,, II	40	19	3	40	14·5	12	16	12	38	31	100
,, III	40	7	1	28	15	14	4	10	65	63	101
Red snapper (Ikan merah)	40	8	1	31	23	16	2	10	65	62	105
Thread fin (Senangin)	40	7	1	28	22	14	5·5	8	62	60	98
Thread fin (Kurau)	100	8	2	27	18	12·5	7	11	63	54	100
Raia livers	40	10	2	30	19	12	3	12	60	58	102
Horse mackerel (flesh)	100	8	1	28	16	12	5	11	62	56	99
Horse mackerel (skins)	100	6	12	20	11	9	4	7	64	61	102
Tunny	80	9	1	26	20	10	5	6	67	61	103
Carp (Twatow)	40	7	1	27	17	12	6	7	63	61	98
Carp (Song Hu)	40	6	1	29	16	12	7	10	67	60	103
Carp heads (Twatow)	100	7	2	24	18	14	2	7	66	60	99

It is clear that no protamine has been obtained from the roes. Fractions I and II of the female and I of the male roes appear to contain proteins

resembling the histones. This may depend upon the maturity of the milt [Kossel and Staudt, 1927]. The flesh muscles show slight differences from the analyses of mammalian tissues in that the total diamino-acid content is generally higher. This appears to be due to the presence of a greater amount of histidine, the most notable exceptions being found in the red snapper and the carp heads. General support of these statements is given by the results with Kossel's method (Table III).

Summary.

1. The diamino-acid content of several fish tissues has been determined by Van Slyke's method, and in a few cases by a modification of Kossel's method.

2. The value for diamino-acid nitrogen in the flesh muscles of fish appears to be higher than that previously obtained for mammalian tissues. This seems to be due mainly to an increased amount of histidine-nitrogen.

3. Some evidence is brought forward showing that incomplete hydrolysis leads to error in the Van Slyke analysis. Different tissues appear to require different lengths of time to ensure complete hydrolysis with 25 % HCl.

4. In the separation of histidine and arginine as silver salts, it appears that unless the mixture is kept slightly acid there is a danger of precipitating some of the arginine together with the histidine.

In conclusion, I should like to thank the Hon. Mr C. F. Green, formerly Director of Fisheries, and Mr W. Birtwhistle, of the Fisheries Department, for kindly supplying much of the material used in this work, and for assistance in choosing and naming the specimens. I am also indebted to my assistant, Mr J. P. Morris, for much help in carrying out some of the manipulations.

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XXI. VITAMIN D FROM STEROLS OF MUMMIFIED EGYPTIAN BRAIN.

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THE stability of ergosterol when present in minute amounts in ordinary cholesterol from all animal sources (brain, gallstones, blood, skin, eggs, etc.) is surprising in view of the relatively labile character of ergosterol itself. This unsuspected contamination of "pure" cholesterol was revealed in the first instance by its antirachitic activity acquired after irradiation, and is confirmed by spectrographic analysis as well as by a specific colour reaction. It is now well established that ergosterol is the specific parent substance of vitamin D [Rosenheim and Webster, 1928, 1, 2]. In explanation of the protective action of cholesterol towards ergosterol, it has been suggested that it possesses an antioxidant function similar to that of the sterols in natural crude rubber [Bills, Honeywell and MacNair, 1928].

There is evidence that this protective action is exerted not only in freshly prepared specimens of cholesterol, but that it remains unimpaired over a long period of years, for some of the specimens of cholesterol, in which the presence of ergosterol was originally discovered, had been prepared from brain 20 years previously [Rosenheim and Webster, 1926]. Further, in a sample of gallstone-cholesterol prepared by Prof. Abel 16 years ago, the presence of ergosterol has been demonstrated spectroscopically and biologically by Bills, Honeywell and MacNair [1928].

Cholesterol and fatty acids have been recovered unchanged from various organs of Egyptian mummies of the pre-dynastic and later periods by Schmidt [1907] and from the brain of a mummy of unknown date by Abderhalden [1911]. Whilst fresh brain contains the whole of its cholesterol in the free state [M. C. Rosenheim, 1914], it has been found by Mair [1913] that as much as 99 % of the cholesterol of ancient Egyptian brain is present in combination with fatty acids (palmitic and stearic) as esters.

Through the kindness of Dr W. Mair we received part of a mummified brain and a specimen of cholesterol prepared from it by him 16 years ago. The brain is of Coptic origin, dating from about 500 A.D., and one of the series described by Ruffer [1913], having been removed from bodies found in tombs in Antinoë in Upper Egypt. No embalming process had been used in this instance and the organs of the bodies were preserved *in situ* through 14 centuries, shrunk and mummified under the influence of the dry atmosphere of Egypt.

We had no difficulty in preparing the pure cholesteryl esters, in a yield of about 12 % from the brain, by employing a simple extraction method and avoiding the use of charcoal on account of its destructive effect on ergosterol. The dry, waxy brain was broken up under water, suspended in an equal volume of 2 % KOH and warmed for a short time on a water-bath. The esters collected as a yellowish oil on the surface of the brown solution and solidified on cooling, giving rise to the characteristic display of colour on passing through the liquid-crystalline phase. After extraction with ether, they crystallised on concentration of the extract. One recrystallisation from ethyl acetate and one from acetone yielded a perfectly white product, M.P. 78–80°, $[a]_{5461}^{16^\circ} - 29^\circ$ (cholesteryl palmitate, M.P. 78–80°, $[a]_D - 24^\circ$, stearate, M.P. 82°).

The crude esters gave the Lifschütz "oxycholesterol" reaction and, when freed from this substance by recrystallisation, showed the typical blue colour with trichloroacetic acid, which is specific for ergosterol [Rosenheim, 1929]. The esters also gave an intense Tortelli-Jaffé reaction (green colour in chloroform solution with dilute bromine), which according to Häussler and Brauchli [1929] is characteristic for ergosterol and is not given by pure cholesterol. Spectroscopic analysis of a 5 % ethereal solution of the esters showed the absorption spectrum of ergosterol in the region 280–290 $\mu\mu$.

Feeding experiments employing our usual technique proved conclusively that daily doses of 2 mg. and 4 mg. of the esters after irradiation (equivalent to 1 mg. and 2 mg. cholesterol) exerted the same antirachitic action as 0·05 γ of irradiated ergosterol. The same result was obtained with 2 mg. doses of the irradiated cholesterol preparation of Dr Mair. We found previously [Rosenheim and Webster, 1926] that doses of from 1–4 mg. of irradiated brain-cholesterol were necessary to prevent rickets in rats kept on a rachitogenic diet and it is therefore evident that the ergosterol content of cholesterol, isolated from brain after about 1400 years, is still of the same order as that of cholesterol prepared from fresh brain.

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XXII. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

V. THE ISOLATION OF *n*-NONACOSANE AND DI-*n*-TETRADECYL KETONE.

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In two of the earlier papers of this series we have described the phosphatides present in the fraction of the ether extract obtained by adding excess of acetone to the ethereal solution. This so-called phosphatide fraction contains in the present case two substances which do not contain phosphorus, and in this paper we describe our investigations into the chemical nature of these compounds which appear in the phosphatide fraction by reason of their insolubility in ether-acetone.

These substances, which may represent nearly one-half of the fraction or one-fifth of the total ether extract, can be readily separated from the true phosphatide material by extraction with boiling acetone, in which they are very readily soluble. On cooling, they separate at once in crystalline form, and it is shown that the main constituents are the paraffin *n*-nonacosane $C_{29}H_{60}$ and the closely allied di-*n*-tetradecyl ketone $C_{14}H_{29}.CO.C_{14}H_{29}$. The presence of such large amounts of nonacosane is surprising, for although higher paraffins are very widely distributed in the plant kingdom, they have been generally isolated in extremely small amounts. Nonacosane has been repeatedly isolated from various plant organs and identified by its melting point, but as Piper and his co-workers [1925] have shown that determinations of melting point and molecular weight are insufficient to define absolutely the number of carbon atoms in the constituent paraffins of paraffin wax, it is probable that the identification of most of these naturally occurring paraffins remains uncertain. At present the number of carbon atoms in such a substance can be determined only by X-ray analysis, and we have to thank Mr S. H. Piper for the final identification of the cabbage paraffin as nonacosane.

The separation of the ketone from the paraffin and its absolute identification as di-*n*-tetradecyl ketone have given great trouble on account of the very similar physical properties of the two substances. It was finally accomplished by taking advantage of the greater solubility of the ketoxime in a mixture of equal parts of light petroleum and acetone, a solvent in which the paraffin is practically insoluble. The regenerated ketone (M.P. 80·5–81°)

was similar in all its properties to synthetic palmitone (M.P. 82–82·5°), but a mixed melting point (76°) showed that the ketones were not identical. Resort was again made to X-ray analysis, and Mr Piper was able to show firstly that the ketone, like the hydrocarbon, contained 29 carbon atoms, and secondly that the carbonyl group was in the centre of the chain. The ketone chain however is so long that he could not be certain whether the ketone was the symmetrical di-*n*-tetradecyl ketone $CH_3.(CH_2)_{13}.CO.(CH_2)_{13}.CH_3$ or the unsymmetrical palmityl myristyl ketone $CH_3.(CH_2)_{14}.CO.(CH_2)_{12}.CH_3$. It seemed not unreasonable to think that the hydrocarbon and the ketone were synthesised in the plant from fatty acids by a reaction in which two molecules of fatty acid condense to yield a ketone, which on reduction would be converted to a hydrocarbon. This may be expressed thus:

$$\begin{matrix} R_1.COOH \\ R_2.COOH \end{matrix} \longrightarrow \begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!CO \longrightarrow \begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!CH_2.$$

If the ketone were palmityl myristyl ketone, then the synthesis in the plant of nonacosane from one molecule of palmitic acid and one of myristic acid—both of which occur in plant products—seemed a likely supposition. On the other hand, if the ketone were the symmetrical di-*n*-tetradecyl ketone, it would be necessary to postulate a synthesis involving two molecules of pentadecylic acid, and it is well known that the naturally occurring higher fatty acids up to and including stearic acid contain an even number of carbon atoms. Although the higher paraffins have been isolated from a large number of plant sources, no evidence has been forthcoming as to their origin in the plant, and the lack of a reasonable hypothesis for their metabolism made it appear vital that the exact position of the carbonyl group should be established with certainty. Accordingly the alternative ketones suggested by Piper were synthesised and a comparison of the melting points left no doubt that the cabbage ketone was di-*n*-tetradecyl ketone. We have at present no evidence of the existence in natural products of pentadecylic acid. It is true that Thörner [1879] isolated the so-called lactaric acid from fungi and that a ketone, lactarone $(C_{14}H_{29})_2 : CO$ (M.P. 81·5–82·5°), was made by Chuit [1889] from this acid. Bougault and Charaux [1912] have since shown, however, that this acid is impure stearic acid, so that the ketone which is quoted in Beilstein as di-*n*-tetradecyl ketone is really impure stearone. Hence the hypothesis that the ketone is formed from two molecules of pentadecylic acid needs modification. It seems reasonable however to surmise that the paraffin and ketone are concerned in the metabolism of fatty acid, and, although we are unable to express the mechanism by which these three types of compound are formed from each other, we are of the opinion that the finding of a paraffin and a ketone of the same number of carbon atoms in the cabbage may prove significant when further information is available.

A search of the literature shows that what we refer to in the experimental part of this paper as the crude hydrocarbon, *i.e.* the mixture of paraffin and

ketone, has been encountered by other workers. Sando [1923] examined the wax-like coating on the surface of apples. One of his crude fractions had the same melting point and gave the same combustion figures as those quoted later for the crude hydrocarbon from the cabbage; from it he isolated a paraffin (M.P. 63·5–64°) which he regarded as $C_{30}H_{62}$. We feel confident also that the ketone $C_{53}H_{106}O_2$ isolated by Hesse [1893] from cocoa leaves and the so-called myristone obtained by Jacobson [1911] from lucerne were likewise mixtures of paraffin and ketone.

EXPERIMENTAL.

220 kg. leaves from winter-sown cabbage picked in May (batch *R*) yielded 220 g. of ether-soluble substances. The fraction insoluble in ether-acetone was extracted with hot acetone and filtered. The investigation of the insoluble phosphatide material has already been discussed [Channon and Chibnall, 1927]. The 35 g. of crystalline material obtained by cooling the hot acetone extracts was greenish in colour and obviously contained some fatty and phosphatide substances. The material was therefore saponified with alcoholic potash, allowed to stand overnight and filtered, the residue being washed with cold alcohol. By recrystallisation from hot acetone there was obtained 30 g. of material which was distilled at 255°/2 mm. The distillate (27 g.) was similar in appearance to, and had the same solubility properties as, paraffin wax. Recrystallised from a large volume of hot acetone, it yielded 23·9 g. of fine silky needles which melted at 63°, although the melt did not clear until a temperature of 67° was reached. The material was only slightly soluble in cold methyl and ethyl alcohols or acetone, slightly more soluble in cold ether, benzene or chloroform, but extremely soluble in hot ether, acetone or benzene. The ethereal solution did not absorb bromine, and did not give the Liebermann-Burchard reaction for sterols. Analysis showed however that although the major part of the material must consist of one or more hydrocarbons, some other substance which contained oxygen was present (found: C, 84·35, 84·28 %; H, 14·38, 14·47 %). The nature of this oxygenated body long eluded us. At the outset, we were deceived by an acetyl value of 12 into devoting considerable time in an attempt to remove higher alcohols, and much material was unfortunately wasted before we obtained clear evidence that the major part of the oxygen was to be ascribed to a ketone.

Determination of melting point. As we have found this determination so helpful in the fractionation of the crude hydrocarbon, we think that a short description of the method employed, which was recommended to us by Prof. Francis, may be of use to other workers in judging the purity of fractions of this type, which so often are isolated from plant products in small amounts only. The temperature of the bath is rapidly raised to within two degrees of the melting point, and then allowed to rise very slowly. A pure paraffin should melt to a clear liquid within the limits of 0·3° and should crystallise

out again in long needles within a temperature fall of 0·5° below the lower of these limits. The processes of melting and setting within these limits are slow, and the temperature of the bath must be held steady for several minutes. The ketone should melt to a clear liquid within a range of 0·5°, and with a nucleus should set crystalline within a temperature fall of 1°. A mixture of the paraffin and ketone becomes a white molten mass at a temperature which may be in some cases only 0·5°, but in others as much as 12–15° below the temperature at which the melt clears. According to our experience, it is only a mixture of the paraffin with the ketone which gives an indefinite melting point of this type; mixed ketones and probably mixed paraffins give sharp melting and setting points. We have no hesitation in saying that the ketone isolated by Jacobson [1911], which softened at 68° and melted at 74–77°, is a mixture, and that its identification as myristone (M.P. 76°) is unwarranted. We have encountered many fractions giving similar melting points and combustion figures during the present research.

Isolation of nonacosane, $C_{29}H_{60}$.

13 g. of the crystalline material mentioned above were submitted to fractional distillation at a pressure of 0·1 mm., and crystallisation from benzene-alcohol. Seven distillations were made in all, and there was finally obtained, from the lower boiling fractions, 1·5 g. of substance crystallising in very thin glistening plates which melted sharply at 62·7–62·8° (uncorr.) and set without a nucleus at 62·6–62·3°.

Analysis. 0·1057 g. substance gave 0·3307 g. CO_2, 0·1387 g. H_2O.

Found: C, 85·33 %; H, 14·58 %.

$C_{29}H_{60}$ requires: C, 85·20 %; H, 14·80 %.

Mr Piper reports on this substance as follows. "Taking λ, K_α Fe = 1·932 Å, main spacing of melted specimen = 38·82 Å. The hydrocarbon contains 29 carbon atoms and the photograph indicates that it is pure." The substance is therefore *n*-nonacosane, $C_{29}H_{60}$.

*Isolation of di-*n*-tetradecyl ketone, $CH_3.(CH_2)_{13}.CO.(CH_2)_{13}.CH_3$.*

The higher boiling fractions after a long fractional crystallisation from benzene-alcohol were finally collected into six samples. Melting point determinations showed that each sample contained nonacosane mixed with one or more other substances, for all became molten around 63°, but the temperatures at which the melts cleared ranged from 63·5 to 75°. It was clear that the procedure adopted was not likely to yield any of these other substances, which analysis showed contained oxygen, in a state of purity. When evidence was finally obtained that one or more of the substances was a ketone, it was found possible to effect a separation by taking advantage of the greater solubility of the ketoxime in a mixture of light petroleum and acetone.

Each of the fractions mentioned above was treated in alcoholic solution with hydroxylamine, the excess of the latter removed, and the product

dissolved in a small volume of light petroleum. An equal volume of acetone was then added and the resulting precipitate filtered off. By repeating this operation on the precipitate, and also on the filtrate, there was finally obtained from each fraction a somewhat crude hydrocarbon melting at 63° and an oxime melting at 50°. The higher melting fractions gave a greater proportion of oxime, and since the products from all the fractions appeared to be similar they were united, and in this way there were obtained 4·5 g. of crude hydrocarbon and 2·2 g. of crude oxime. The latter was dissolved in 100 cc. of warm glacial acetic acid and cooled on ice. 0·44 g. of hydrocarbon crystallised out in fine needles. These were filtered off, and to the warmed filtrate an equal volume of warm absolute alcohol was added. On cooling, the major part of the oxime came out as fine flakes. These were dissolved in 30 cc. of warm glacial acetic acid and 0·1 g. of crude hydrocarbon separated by cooling as before. The filtrate was evaporated to dryness *in vacuo*, dissolved in ether, the ethereal solution washed to remove traces of acetic acid and again evaporated to dryness. Yield of oxime 1·5 g.; M.P. 50-51°.

Analysis. 0·0915 g. substance gave 0·2682 g. CO_2, 0·1050 g. H_2O.
0·1728 g. substance gave 5·0 cc. of moist nitrogen at 18° and 752 mm.

Found: C, 79·91 %; H, 12·88 %; N, 3·29 %.
$C_{29}H_{58}NOH$ requires: C, 79·63 %; H, 13·50 %; N, 3·21 %.

The ketone was regenerated from the oxime by boiling for some hours in alcohol saturated with hydrochloric acid. By repeated crystallisation from benzene-alcohol, there was obtained 0·7 g. of ketone crystallising in thin glistening plates. M.P. 80·5-81°; setting point 80·5–80°.

Analysis. 0·0832 g. substance gave 0·2487 g. CO_2, 0·1043 g. H_2O.
Found: C, 82·45 %; H, 13·93 %.
$C_{29}H_{58}O$ requires: C, 82·37 %; H, 13·87 %.

The melting point of this substance suggested that it was palmitone, and accordingly a sample of this ketone was synthesised from pure palmitic acid by Kipping's [1890] method. It melted at 82·2–82·5° and set crystalline at 81·7–82°, confirming the value (82°) given by Kipping and not that (76°) quoted by Saville and Shearer [1925]. A mixture of this palmitone with the cabbage ketone melted sharply at 75·8–76·2° and set crystalline at 75·5–74·5°, showing that the two ketones were not identical. Mr Piper then kindly submitted the cabbage ketone to X-ray analysis and reported as follows. "Main spacing 38·8 Å. The melted specimen showed only side spacings, but when crystallised and rubbed gave a perfect photograph. The even orders 2 and 4 are just visible, the odd orders 1, 3, 5, 7, 9 give the above spacing. The ketone contains 29 carbon atoms and is probably $C_{14}H_{29}.CO.C_{14}H_{29}$, since the oxygen atom appears to be half-way along the chain. The chain is so long however that the possibility of the ketone being $C_{15}H_{31}.CO.C_{13}H_{27}$ is not excluded." Both these ketones were accordingly synthesised. The

synthetic di-*n*-tetradecyl ketone melted at 80–80·5° and set crystalline at 79°. Mixed with the cabbage ketone it melted at 80·5–81° and set crystalline at 79°. The synthetic palmityl myristyl ketone melted at 74–74·5°.

Reviewing our experience on the isolation of these two substances from the crude hydrocarbon fraction, there is no doubt that much time and trouble would have been saved if the product obtained by distillation after the initial saponification had been oximated and the oxime removed as described above. The long fractionation actually carried out however has served to show that the chief constituents are nonacosane and di-*n*-tetradecyl ketone, and that they are present in the ratio of three of the former to one of the latter. Other products are present, but they appeared in fractions too small to warrant the hope that pure compounds could be isolated from them.

*Synthesis of di-*n*-tetradecyl ketone*, $CH_3.(CH_2)_{13}.CO.(CH_2)_{13}.CH_3$.

Kipping's [1890] method for the preparation of palmitone was used. 2 g. of pentadecylic acid (M.P. 52°), for which we have to thank Prof. W. E. Garner, were heated in a small beaker in a metal-bath at 205–210° and phosphorus pentoxide was added slowly, in small portions with constant stirring. The reaction was complete in about 5 minutes, when the molten mass was poured into water to decompose the excess of phosphorus pentoxide. Sodium hydroxide was then added to convert the excess pentadecylic acid to soap and the mixture raised to the boiling point. On cooling, the ketone and other resinous products solidified at the surface as a waxy cake, which was removed and washed in the usual way. The ketone was separated from the other products by repeated extraction with small volumes of hot 90 % alcohol. The crude ketone was dissolved in 90 % alcohol, and the solution boiled with charcoal and filtered. On cooling the ketone separated in thin glistening plates. Yield 0·2 g.; M.P. 78·5–79°. Recrystallised twice from benzene-alcohol it melted sharply at 80–80·5°, and with a nucleus set crystalline at 79°.

Analysis. 4·920 mg. substance gave 14·840 mg. CO_2, 6·05 mg. H_2O.
Found: C, 82·30 %; H. 13·67 %.
$(C_{14}H_{29})_2CO$ requires: C, 82·47 %; H, 13·74 %.

Synthesis of palmityl myristyl ketone, $CH_3.(CH_2)_{14}.CO.(CH_2)_{12}.CH_3$.

We have to thank Prof. R. Robinson for suggesting this synthesis, which is similar to that of lactarinic acid, 6-ketostearic acid [Robinson and Robinson, 1925].

Ethyl a-dodecylacetoacetate. Ethyl laurate, obtained by the fractional distillation of the ethyl esters of the fatty acids of coconut oil, was reduced to lauryl alcohol by means of sodium and dry ethyl alcohol at 130°. The alcohol was converted to dodecyl iodide by heating with iodine and red phosphorus. Sodium (1·8 g.) was dissolved in 30 cc. absolute alcohol, 15 g. of ethyl acetoacetate were added and finally 22·5 g. dodecyl iodide. The mixture was boiled

gently for $3\frac{1}{2}$ hours, cooled, poured into water and the mixture extracted with ether. The oil resulting from evaporation of the solvent was fractionally distilled *in vacuo*, and the higher boiling fraction again distilled. The fraction (12·5 g.) collected at 184–187°/8 mm. analysed as follows:

0·1380 g. substance gave 0·3667 g. CO_2, 0·1442 g. H_2O.

Found: C, 72·44 %; H, 11·61 %.

$C_{18}H_{34}O_3$ requires: C, 72·50 %; H, 11·41 %.

Palmityl myristyl ketone. 0·9 g. sodium was emulsified under toluene, washed with dry ether and then suspended in 75 cc. of dry ether. 12·1 g. of ethyl α-dodecylacetoacetate dissolved in dry ether were added and the mixture slightly warmed until all the sodium had dissolved. It was then cooled on ice and 12·2 g. palmityl chloride in 20 cc. dry ether were added. The mixture was allowed to stand for 1 hour and then boiled for 10 minutes. The sodium chloride was removed by washing with water and the ether removed by evaporation. The resulting ethyl $\alpha\alpha$-dodecylpalmitylacetoacetate was an oil which readily crystallised on standing. The product was dissolved in 300 cc. of cold alcohol and as much 5 % aqueous potassium hydroxide added as possible without causing precipitation (14 cc.). After standing for 5 days to effect the slow hydrolysis of the carbethoxy-group, sufficient strong aqueous potassium hydroxide was added to make the concentration 5 %, and the solution boiled for 2 hours. The hot solution was then poured into an excess of boiling water, and after cooling the ketone was removed by extraction with ether. The ethereal solution was concentrated to about 180 cc. and 2 volumes of acetone were added. The palmityl myristyl ketone was almost completely precipitated. It was redissolved in hot acetone and the solution clarified by boiling with charcoal. On cooling, the ketone separated in fine silky plates. Yield 4·5 g.; M.P. 73–74°. Recrystallised twice from equal volumes of alcohol and benzene, it was obtained in large lustrous plates melting at 74–74·5° and setting crystalline at 73°. The melting point was unchanged on further recrystallisation.

Analysis. 4·892 mg. substance gave 14·770 mg. CO_2, 6·03 mg. H_2O.

Found: C, 82·33 %; H, 13·70 %.

$C_{15}H_{31}.CO.C_{13}H_{27}$ requires: C, 82·47 %; H, 13·74 %.

After the removal of the palmityl myristyl ketone, the acetone-ether filtrate was evaporated to dryness and submitted to steam distillation. The distillate was filtered, and from the residue, after crystallisation from aqueous acetone, there was obtained 2·7 g. of methyl tridecyl ketone; M.P. 38·5–39°.

We wish to express our thanks to Mr S. H. Piper, of the University of Bristol, for the interest he has taken in this work and for submitting a number of specimens to X-ray analysis. One of us (A. C. C.) is indebted to the Royal Society for a grant from which part of the expenses of this research was defrayed.

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XXIII. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

VI. SUMMARY AND GENERAL CONCLUSIONS.

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In a series of papers [Chibnall and Channon, 1927, 1, 2, 3; Channon and Chibnall, 1927], we have recorded certain results which we have obtained in the analysis of the ether extract of the leaf-cell cytoplasm of the cabbage. The object of the present paper is to discuss these results so far as is at present possible from their chemical and physiological aspects, and, in passing, to refer to our investigations of the pigments and of the unsaturated portion of the unsaponifiable fraction of which no account has yet been given.

For simplicity in presentation, we propose to confine all further remarks on this investigation to batch *R*, consisting of 220 g. of ether extract prepared from 220 kg. of cabbage leaves. By methods already described, this extract was separated into the following fractions.

A. The fraction precipitated by acetone, which was further subdivided into

   I. The fraction insoluble in hot acetone, wt. 57·8 g.

   II. The fraction soluble in hot acetone, ,, 35·0 g.

B. The traction soluble in acetone-ether, ,, 119·9 g.

   Saponification of an aliquot part showed that this fraction contained

   III. Fatty acids soluble in light petroleum, wt. 38·3 g.

   IV. Unsaponifiable matter ,, 48·6 g.

   V. Acidic products insoluble in light petroleum, consisting of chlorophyll derivatives and possibly hydroxy-acids.

As a preliminary to the further study of fractions (IV) and (V) it was necessary to determine the chlorophyll content of the original ether extract.

Determination of chlorophyll and carotenoids.

Extensive investigations have been made by Willstätter and his colleagues [Willstätter and Stoll, 1913] into the chlorophyll content of leaves; we have therefore thought it sufficient to determine the total chlorophyll content only, and not the α- and β-chlorophyll separately. Willstätter's method is to shake the ether extract with methyl alcoholic potash and to compare the green colour of the resulting phyllins with that given by a specimen of pure chlorophyll submitted to similar treatment. This method was applied to the

ether extract of the cabbage but the green tint of the resulting phyllins did not match that given by a specimen of pure chlorophyll. This suggested that during the coagulation of the cytoplasm by heat part of the magnesium had been removed from the molecule. Accordingly the ethereal solutions of the cabbage extract and of pure chlorophyll were first shaken with an aqueous solution of oxalic acid to remove the magnesium and so convert the chlorophyll to phaeophytin. The resulting solutions of phaeophytin were then submitted to Willstätter's treatment with methyl alcoholic potash and an aqueous solution of the complex potassium salts was prepared. The green tints of these were easily comparable and subsequent calculation showed that the chlorophyll content of the acetone-ether fraction B of batch *R* was 9·3 %.

The carotenoids were determined at the same time by Willstätter's method. The acetone-ether fraction B contained 0·86 % carotene and 1·39 % xanthophyll, giving a ratio $\frac{\text{carotene}}{\text{xanthophyll}} = 0{\cdot}62$, which is of the same order as that found by Willstätter for the pigments of the whole leaf. On the other hand, the ratio $\frac{\text{chlorophyll}}{\text{carotene} + \text{xanthophyll}}$ is 7·7, which is about twice that found by Willstätter for whole leaves. Two explanations of this high value suggest themselves: (*a*) these pigments are not located solely in the leaf cell cytoplasm; (*b*) the colours of the pigments had faded somewhat before they were quantitatively determined.

Unsaturated unsaponifiable matter (Fraction IV).

106·2 g. of the acetone-ether filtrate B yielded 42·5 g. of unsaponifiable matter, a brown gummy mass having iodine value 105 and acetyl value 168, and containing 21·8 % of sterol precipitated by digitonin. Present in this fraction also are the lipochrome pigments, *i.e.* 0·92 g. carotene and 1·48 g. xanthophyll, together with 6·13 g. phytol derived from the chlorophyll and present in the extract. The fraction contained in addition that portion of the hydrocarbon fraction which remained in solution when the original ether extract was precipitated with acetone. It is clear that the effective fractionation of a mixture of substances in the presence of higher alcohols and sterols having various similar solubility properties is no easy matter. Repeated attempts to obtain pure fractions by crystallisation from a variety of solvents left us convinced that we were obtaining products contaminated by the hydrocarbon, which we were unable to remove by this method. It was decided, therefore, to submit the material to fractional distillation *in vacuo*; a liquid fraction B.P. 175° to 185°, and a small fraction B.P. 222° to 240° at 2 mm. pressure were removed in this way. The first fraction by careful purification was shown to consist mainly of phytol, whilst the second was crude hydrocarbon (M.P. 63°–65°). Attempts to distil the remainder, however, resulted in marked decomposition, as was shown by the nature of the products obtained and by the fact that the residue failed to give any precipitate with digitonin. From this distillate there were isolated white crystalline products

of various melting points, the highest observed being 129°. This latter fraction was shown by the digitonin method to contain only 82 % of sterol. In view of these difficulties and the lack of sufficient material for a large scale fractionation, we were reluctantly compelled to leave over the question of the nature of these higher alcohols and sterols until a larger amount of material was available.

General summary.

We now propose to discuss what we consider the essential results of these researches in both their chemical and physiological aspects.

The following table shows the results which we have obtained in the analysis of a typical sample of cabbage-leaf cell cytoplasm. It is admittedly incomplete in some respects and must remain so until material can be obtained in still larger bulk. During the course of this work some 10 cwts. of cabbage have been used and a description of some of the difficulties and uncertainties which we have encountered in what appears to be the first attempt to study such an ether extract is given in the footnotes to the table for the guidance of any who may wish to extend these results.

The methods used in the separation of the various fractions have already been discussed.

Table I. *Summary of the ether-soluble substances of cabbage-leaf cytoplasm.*

(Batch *R*, consisting of 220 g. of material.)

	Wt. g.	% of total material
Pigments		
Chlorophyll (a and β) ...	20·5	9·3
Carotene ...	1·1	0·5
Xanthophyll ...	1·7	0·8
Substances containing phosphorus		
Calcium phosphatidate ...	40·6	18·4
Unidentified calcium salts, possibly of fatty acids and phosphoric acid	11·3	5·0
Unidentified iron compound ...	6·7	3·0
Glycerides and waxes		
Containing palmitic, stearic, linolic and linolenic acids ...	38·3	17·5
Glycerol ...	2·8	1·3
Unsaponifiable matter		
Saturated fraction, chiefly nonacosane and di-*n*-tetradecyl ketone ...	27·0	12·3
Unsaturated fraction		
Sterols (by digitonin) ...	9·8	4·5
Unidentified products, probably alcohols and hydrocarbons ...	29·2	13·3
	189·0	85·9

Note 1. *Cause of losses.* It will be observed that the percentage total of the fractions is only 85·9 and that there has been an apparent loss of 14·1 %. In considering these losses it may be pointed out, firstly, that about one-third of this loss is entailed by the use of methods necessary for purifying the hydrocarbon fraction (36 g. of crude product gave only 23 g. of purified product), and, secondly, the large number of manipulations in obtaining the various fractions has of necessity caused considerable mechanical losses, which cannot be avoided when working with fatty materials. Even so, there

remains the probability that about 5 % of the total material is unaccounted for, and a similar loss has been encountered in every batch. As to the reasons for it, we have little information. It always occurs as the result of the separation of the acetone-ether-soluble fraction into fatty acids, unsaponifiable matter and chlorophyll degradation products, and the material remains in the aqueous phase after extraction of the fatty acids. This fact suggests that there may be present an ether-soluble substance which is destroyed on treatment with alcoholic alkali, or alternatively that acidification of the soap solution after the removal of the unsaponifiable fraction has resulted in the precipitation not only of fatty acids and acidic chlorophyll products, but also of other acids sparingly soluble in ether. Although the acidified solution was extracted many times with ether, it was found necessary that the aqueous solution should be very dilute in order to facilitate extraction, and hence any substance, soluble to a limited extent only in ether and water, would remain to some extent in the aqueous phase. Such substances might be hydroxy-acids.

Note 2. *Unidentified iron compound.* As we mentioned in an earlier paper, only about 1 g. of this curious substance was isolated. (Found C = 52·4 %, H = 8·1 %, P = 5·3 %, Fe = 8·9 %. Nitrogen is absent.) We are not yet in a position to give any further information. In the table the gross amount of this substance has been computed from the iron content of the calcium phosphatidate. This is greatly in excess of the amount actually isolated, but it must be remembered that we treated this material as an incidental impurity and removed it whenever possible when purifying the calcium phosphatidate.

Note 3. *Residual ether-soluble phosphorus.* The ether-soluble phosphorus present as calcium phosphatidate represents about 60 % of the whole. In a previous paper [Chibnall and Channon, 1927, 2] it was suggested that the remainder was present as the calcium salt of an easily hydrolysable monoglyceridephosphoric acid, because on shaking the ether-soluble phosphorus compounds in ether solution with mineral acid part of the phosphorus passed into the aqueous layer and fatty acids appeared in the ether solution. Our subsequent investigations have tended to weaken this view for two reasons. Firstly, the iron compound which contains 5 % of phosphorus and no calcium represents about one-tenth of the ether-soluble phosphorus fraction. Since the ratio of calcium to phosphorus in this fraction was originally unity, it is clear that there must have been present some calcium which was not in combination with phosphorus and that the unity ratio was merely fortuitous. Secondly, shaking the original ether-soluble phosphorus solution with mineral acid removed part of the phosphorus as phosphoric acid and not, as we had thought, as glycerophosphoric acid. Further, determinations of glycerol on the phosphatide fraction have shown that the amount of glycerol in the fraction is no more than is required by the calcium phosphatidate present. Hence the fatty acids which appeared in the ether layer cannot have originated as the result of the hydrolysis of a monoglyceridephosphoric acid. We have

obtained no further information as to the chemical nature of the compounds giving rise to the phosphoric acid and fatty acids on shaking with mineral acid; and this question remains therefore undecided at present. We incline to the view that the sources of these substances are calcium soaps and calcium phosphate, and that only 60 % of the ether-soluble phosphorus represents phosphatides.

Note 4. *Glycerides and waxes.* Estimations of glycerol by the methods of Zeisel and Fanto [1903] on the acetone-ether-soluble material of batch *R* gave a ratio of $\frac{\text{fatty acids}}{\text{glycerol}} = 7 \cdot 8$, slightly higher than that recorded for batches *G* and *M* in a previous paper. This method in our hands has given a theoretical yield of glycerol from barium glycerophosphate, but we have recently found that it gives very low yields from distearin and tristearin. The method of Fanto [1904] was found to give a theoretical yield with tristearin, and applied to batch *R* gave a ratio $\frac{\text{fatty acids}}{\text{glycerol}} = 13$. The original method, which has the advantage of requiring only 0·2 g. of material, is clearly inapplicable to these complex plant extracts, possibly on account of the unidentified substances mentioned under note 1 above. Tristearin gives a ratio of $\frac{\text{fatty acids}}{\text{glycerol}} = 9 \cdot 3$, so that about one-third of the fatty acids present in the acetone-ether-soluble material of batch *R* was present as substances other than glycerides, probably as esters of higher alcohols.

General discussion.

The yield of the chief constituents.

By methods which were referred to in the first paper of this series [Chibnall and Channon, 1927, 1] we calculate that the total amount of ether-soluble material in cabbage-leaf cytoplasm is about 3·5 % of the total leaf solids. This figure may not represent the total amount of these substances present in the leaf, because our preparations of coagulated cytoplasm were free from cell-wall material which, in consequence, has not been ether-extracted. The variations which we have found in the amounts of the chief constituents are given in Table II.

Table II. *Ether-soluble substances. Variation in chief constituents.*

	% of total ether-soluble material	Estimated % of total leaf solids
Total ether-soluble substances	—	3·5
Calcium phosphatidate	9–18	0·3–0·6
Fatty acids in glycerides and waxes	19–27	0·6–0·9
Saturated hydrocarbons and ketones	8–13	0·3–0·5
Other unsaponifiable material	16–25	0·6–0·9

As the presence of fat-soluble substances containing calcium and iron has not been demonstrated hitherto, it appeared to us to be of interest to calculate roughly what proportions of calcium, phosphorus and iron of the cabbage

leaf are present in the form of calcium phosphatidate and the iron compound. The results of our analyses of the cabbage leaf for these inorganic materials are given in Table III.

Table III. *Distribution of Ca, Fe and P in the cabbage leaf, (% of total leaf ash).*

Vol. 23, p. 181, Table III, Fe_2O_3, whole leaf, ***instead of*** **12·9** ***read*** **2·9.**

			ter-insoluble	Ether-soluble
		- -	4·6	0·45
Fe_2O_3	12·9	—	2·9	0·15
P_2O_5	16·0	11·8	4·2	0·73

Water-soluble iron was too small to be determined gravimetrically as Fe_2O_3. Hoagland and Davies [1923] in a discussion of the cell sap of higher plants emphasise the fact that for a proper understanding of the metabolism of such plants it is necessary to gain some insight into the forms in which inorganic elements are held by the plant. They have analysed different plants (species not given) at various stages of growth for water-soluble inorganic elements, and have found that in practically every case all of the potassium is soluble in water as well as the major portion of the other elements, with a few exceptions in the case of calcium. We determined total ash and ether-soluble ash on samples of 300 g. of fresh leaves. As the ether-soluble calcium, iron and phosphorus are computed from figures obtained from the analysis of 220 kg. of fresh leaves, it is obvious that the two sets of figures given in the table are not strictly comparable. They show, however, that about one-twentieth of the total leaf calcium, phosphorus and iron is ether-soluble, and that about one-tenth of the water-insoluble calcium can be ascribed to calcium phosphatidate.

Phosphatides.

One of the most interesting results of these researches has been the discovery of the existence in the cytoplasm of calcium phosphatidate, and experiments are clearly necessary to determine its relation to the glycerides. Thus in a plant which is being starved, does utilisation of this phosphatide and of the glycerides take place? This question of the utilisation of fat by a starving plant is one which has suffered severe neglect because the ratio of carbohydrate to fat was thought to be so high as to make it appear that a study of fat from the tissue was a matter of minor importance. It will be of interest, therefore, to see whether utilisation of the fatty acids, either of the glycerides or of the phosphatide, occurs, and if there is a decrease in the fatty acids it will be necessary to determine whether the fatty acids utilised are those of the phosphatide or of the glycerides. One is naturally tempted to compare these substances with those present in animal tissue, and in this connection the high degree of unsaturation, not only of the phosphatide but also of the glycerides, is interesting. It will be remembered that the fat of adipose tissue is saturated relatively to that of such organs as the liver and that during

starvation this adipose fat is utilised, while that of the liver persists even if death from inanition occurs. The conclusion drawn from these results is that the liver fat, consisting of unsaturated phosphatide, cannot be utilised without causing destruction of the cells of which it is an integral part. Hence, if calcium phosphatidate is fulfilling in the leaf the rôle which lecithin and the other phosphatides play in the animal, we should expect that on starvation the plant would utilise the glycerides only and that the calcium phosphatidate would remain constant in amount. Experiments on this subject are in progress; it is clear that, if calcium phosphatidate is as vital a constituent of the leaf as lecithin is of the animal, it should be present in the leaves of all plants. This question has not been systematically investigated by us yet, but some experiments with spinach suggest that there is a definitely smaller percentage of calcium phosphatidate in spinach leaves than in those of the cabbage.

The literature on plant phosphatides, although extensive, is very unsatisfactory on account of the great difficulties encountered in freeing phosphatidic substances from sugars, amino-acids, etc., and it gives the impression that very little definite information as to the nature of plant phosphatides is available. However, the results of Levene on soya-bean meal show that lecithin is present in that material. This opens up the question as to the physiological relationship existing between the phosphatides of the seed and of the leaf, and it will be interesting to investigate whether such a plant as soya bean, having lecithin and cephalin in its seed, has the calcium or some other salt of phosphatidic acid in its leaves, and, if so, the reasons why storage products should differ from those of physiologically active tissue. Such a finding as the occurrence of different phosphatides in the leaves and seeds would be totally unexpected if we regard the phosphatide as an essential constituent of the cell. On the other hand, such a finding might be understood if the rôles of the two types of phosphatide were different either in their effects on permeability or as agents in the transport of fat. Any view which we can express on these subjects at the present time must be speculative, but the fat-solubility of the calcium salt of phosphatidic acid and the water-solubility of its sodium salt suggests that these phosphatides may be in part responsible for the alteration in cell permeability which can be brought about by changes in the proportion of sodium and calcium ions.

The fatty acids.

A point of interest regarding the fatty acids is that the iodine value of the glyceride fatty acids is about 200 compared with 137 of the fatty acids present in the phosphatide. This higher degree of unsaturation of the glyceride fatty acids is the reverse of the findings in the fats of animal tissues, for in the latter the fatty acids of the phosphatides are considerably more unsaturated than those present in the neutral fat.

As to the question of unsaturation, the suggestion of Leathes and Raper [1925] that the temperature at which fats are formed in plants is one of the

features which determine the degree of unsaturation of the fatty acids is of interest. Recently Terroine *et al.* [1927] and Pearson and Raper [1927] have shown that the degree of unsaturation of the fatty acids present in *Aspergillus niger* grown at temperatures varying from 17° to 35° decreases with a rise of temperature. If such a finding can be applied to a higher plant, it would be expected that there would be a variation in the degree of unsaturation of the fatty acids of autumn- and spring-sown cabbage. The former are picked in April-May and will be referred to as "winter-grown," while the spring-sown plants which are gathered in July-August will be referred to as "summer-grown." Actually, we have found no significant change in the degree of unsaturation of the fatty acids of the winter- and summer-grown cabbages, the extreme values being 195 and 206, the value for batch *D*, as shown in Table I of a previous paper of this series [Chibnall and Channon, 1927, 1], being invalidated by the fact that in the early preparations we had been unable to free the fatty acids completely from chlorophyll degradation products.

Table IV. *Comparison of glyceride fatty acids and crystalline crude hydrocarbon (% of total ether-soluble material).*

	Autumn-sown (picked April–May)		Spring-sown (picked July–August)		
	E	F	H	M	P
Wt. of crude hydrocarbon (*a*)	11·0	10·9	19·0	17·6	16·0
Wt. of glyceride fatty acids (*b*)	23·1	23·0	15·0	16·3	16·6
Iodine value of fatty acids	203	201	206	—	195
Calculated iodine value of (*a*) and (*b*) mixed	137	136	91	—	99

This finding leads us to wonder whether the hypothesis of Leathes and Raper regarding the variation of the iodine value of fatty acids with temperature may not need extension to include not only fatty acids but other fat-soluble substances present. It is to be remembered that the unsaponifiable matter of a leaf fat constitutes a large proportion (up to 40 %) of the whole fat and if variation in the liquidity with temperature is to be expected it seems possible to us that a variation of the degree of unsaturation of the unsaponifiable matter as well as of the fatty acids may be anticipated. Our reason for this is that the amounts of crude saturated hydrocarbon, M.P. 63°, present in our extracts have shown considerable variation with the season of picking, as is shown by Table IV, in which it is seen that 11 % of hydrocarbon is present in winter-grown cabbage, whereas between 16 % and 19 % is found in the summer-grown plants. At the same time, the percentage of fatty acid fell from 23 % to 16 % with no alteration in the iodine value. We admit that the unsaturated portion of the unsaponifiable matter may also require consideration in this connection, but we tend to regard the hydrocarbon as being concerned in the metabolism of fatty acids and being in its physical properties closely akin to the saturated acids. If this hypothesis be

accepted, namely, that the hydrocarbon fraction may be regarded from the point of view of liquidity as saturated fatty acid, calculation shows that the iodine value of the hydrocarbon-fatty acid mixture in the winter-grown cabbage fat is 136 as against 91 for the summer-grown cabbage. Whether this variation in the fat derived from cabbages grown in winter and summer respectively is significant must await further investigation.

We will conclude this paper by referring to a matter to which Leathes and Raper have already drawn attention, namely, the use of the word "fat" in biological chemistry. They have pointed out that much time and labour have been lost in researches in which attempts have been made to follow the metabolism of "fat" by the determination of the amount of ether-soluble material which can be extracted from tissues under various conditions, and they have emphasised the necessity of the determination of the amount of the fatty acids present in the ether extract rather than of the amount of the ether extract itself. Our experiences in this work on the ether extract of cabbage-leaf cell cytoplasm illustrate this point vividly, for the fatty acids make up only about 27 % of the material soluble in ether, and there are present not only calcium phosphatidate and an iron compound containing phosphorus, but also a higher paraffin and ketone, together with unidentified alcohols; in addition, the ether-soluble pigments are present in the extract. In biochemistry, it is frequently necessary, after drying a tissue suitably, to extract it with ether or some fat solvent in order to obtain one particular fat-soluble substance. Such an ether extract as in the present case contains so many and various materials, that it seems desirable to us that some word be adopted merely to denote such an ether extract irrespective of what it may contain. This seems to us more advantageous than the adoption of the word "lipide" which is used by Bloor [1925] to denote a variety of substances soluble in fat solvents and related to fatty acids as esters or potential esters, for nonacosane and di-*n*-tetradecyl ketone may be as intimately concerned in the metabolism of fatty acids as the higher alcohols which are included in such a classification.

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XXIV. THE DIALYSIS OF SMALL VOLUMES OF SERUM UNDER STERILE CONDITIONS.

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It has been found necessary during researches into the colloidal properties of blood-serum and of the component serum-protein fractions to have recourse to methods involving dialysis.

It was found [Voge, 1928], when dealing with syphilitic serum, that the "syphilitic substance" could not satisfactorily be located in either the euglobulin or the pseudoglobulin fraction when Liefmann's method of "splitting" serum was adopted and that part appeared to exist in both fractions. Satisfactory results were obtained however when serum was placed in a collodion membrane and dialysed, the whole of the "syphilitic substance" being located in the fraction insoluble in water. In the method originally employed 10 cc. of serum were examined and dialysis had perforce to be continued for a considerable time to rid the serum of its salts, failing which complete separation was not obtained. This prolonged dialysis introduced other difficulties—increase in the fluid volume and bacterial contamination. These difficulties could be overcome by the use of a manometer and some antiseptic respectively, but it was considered feasible to attempt other methods involving smaller volumes of fluid and dialysis under sterile conditions, as the addition of antiseptics to serum may involve changes which will vitiate the experiment.

Methods involving electrodialysis have also been attempted, but great difficulty was found in regulating the temperature and so this method was abandoned.

A similar line of investigation has been adopted in order to determine in which serum fraction agglutinins can be isolated and it has been shown that they exert their influence in the euglobulin fraction [Voge, 1929], a view which is in agreement with that advanced by Rosenholz [1928], and also by Kroeger and Hektoen [1927], the latter investigating the precipitin phenomenon. These authors state however that hydrolysis takes place and that the active fraction is liberated into the fraction soluble in water.

It is thus apparent that a simple and rapid method of dialysis under conditions as nearly as possible approaching sterility is required, especially in view of the use of such euglobulin-antibody fractions in prevention and treatment of disease.

The work of Rosenholz [1928], Cambessédès and Cochez [1928] and Voge [1929] indicates that upon these lines there will be ground for a revaluation

of our present methods of preventive and curative medicine, since it has already been shown [Rosenholz, 1928] that such a fraction can be readily obtained and kept in a dry sterile condition.

Experimental.

The semi-permeable membranes are prepared from a solution which is made up as follows. 5 g. of pyroxylin are dissolved in 80 g. anhydrous ether and 20 g. absolute alcohol. This syrupy solution must be free from bubbles and must be kept in a wide-necked flask fitted with a rubber stopper. A Wassermann test-tube, 3″ × ½″, cleaned with aqua regia, washed thoroughly in water, alcohol and finally ether and dried, is used. It is necessary to observe these precautions or otherwise the membrane will adhere too firmly to the glass walls and cannot be separated without tearing. The tube is now filled with the pyroxylin solution, inverted, and the contents are allowed to drain. This process is repeated after the first coat has dried. Two coats of such a solution have been found to yield a membrane of sufficient strength and yet thin enough to allow rapid dialysis. The tube is now filled with sterile water and the sac gently separated from the glass walls with a blunt-pointed instrument.

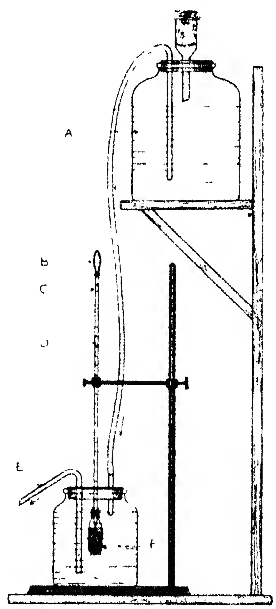

Fig. 1.

A. Sterile distilled water
B. Rubber teat.
C. Plug of cotton wool, sterilised with pipette.
D. Sterile 1 cc. pipette.
E. Outlet pipe dialysing apparatus
F. Sac, prepared as described in the text, bound to pipette so that the point is below the level of the fluid. At the end of 18 hours the solid water-insoluble euglobulin is observed at the foot of the sac.

A sterile 1 cc. pipette and rubber teat are now employed to withdraw 1 cc. of the serum to be dialysed. The point of the pipette is inserted within the mouth of the sac, the contents ejected and the tip of the pipette lowered until it is just below the level of the serum. In this position the sac is firmly bound to the pipette with linen thread and the pipette and sac are placed within a sterile bottle. The stopper fitted to the flask should be of rubber bored with three holes, one for the inflow of sterile water, the second for the pipette and the third for the outflow. It is an additional simplification if the stopper is already fitted to the pipette before the sac is fitted. The pipette and teat in the position indicated act as a manometer and so control the volume of liquid.

At the end of 18 hours a heavy flocculum, insoluble in water, is observed at the bottom of the sac; this is mixed with the supernatant fluid and the contents are withdrawn into the pipette by means of the teat. (Sterile pipettes are fitted with a plug of cotton wool at the upper end so that the teat can be

removed to relieve the pressure and obtain suction.) The contents of the pipette are ejected into a sterile centrifuge tube and the solid removed from the liquid by centrifuging and decanting the supernatant fluid. The water-insoluble protein remaining is now emulsified in sterile saline and can be used for animal experiments.

Isolation of such material has successfully been carried out along these lines and subsequent injections into animals have been performed without complications due to bacterial contamination.

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XXV. THE INFLUENCE OF VARIOUS SUBSTANCES ON LIPASE ACTION[1].

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(Received February 28th, 1929.)

Rosenheim [1910] has investigated the influence of various compounds on the hydrolysis of olive oil by pancreatic lipase. Although no numerical data are submitted, he states that lecithin is inert towards lipase and also that cholesterol retards lipase action. In a previous paper [Corran and Lewis, 1928] results of experiments were recorded which demonstrated that lead, in both the ionic and colloidal form, is capable of increasing the lipolytic augmentation produced by serum, both normal and cancerous. In the present paper, results are submitted on the influence of the chlorides of sodium, potassium and calcium, and of purified lecithin, cholesterol, lead and copper salts on lipolysis in the absence of serum, and the effects of cholesterol, copper and mercury on lipolysis in presence of serum.

The method employed was identical with that outlined in an earlier paper, namely, mixing the glycerol extract of lipase, serum, water (or salt solution) and olive oil emulsion, the latter consisting of 4 parts of purified olive oil to 1 part of 1 % aqueous sodium oleate, and allowing the reaction to proceed at 37° until equilibrium was attained.

I. The influence of lecithin and cholesterol on lipolysis.

As mentioned previously Rosenheim found that lecithin is inert towards lipase, but that its products of hydrolysis are strong augmentors. In the present work a purified lecithin has been employed, in order to ensure that any effect observed is due to lecithin, and not to impurities[2]. A fine suspension in water was used, of strength 0·01 M. Table I gives the mean results obtained.

It is evident that the augmentative effect due to lecithin is small compared with those produced by serum and sodium oleate (for example, 1 cc. of serum and 1 % sodium oleate give augmentations of 20 cc. and 13 cc. respectively [Corran and Lewis, 1928]), the greatest effect being observed with 1 cc. 0·01 M lecithin suspension. Incidentally, the results recorded in Table I confirm the conclusions reached by Rosenheim, that the effect of lecithin on lipase is negligible.

[1] An investigation carried out on behalf of the Liverpool Medical Research Organisation; Director, Prof. W. Blair Bell.

[2] The writer's thanks are due to Mr H. I. Price, of the Dept. of Physical Chemistry, University of Liverpool, for the sample of pure lecithin, prepared by Levene's method [1927].

Table I.

Duration of experiment, 48 hrs. Temperature 37°.

Lipase extract cc.	0·01 M lecithin cc.	Water cc.	Olive oil emulsion cc.	$N/10$ NaOH required cc.	Augmentation in $N/10$ NaOH cc.
1	0·0	5·0	5	27·90	—
1	0·5	4·5	5	28·20	0·30
1	1·0	4·0	5	34·10	6·20
1	2·0	3·0	5	33·80	5·90
1	3·0	2·0	5	31·20	3·30
1	5·0	0·0	5	30·00	2·10

Considering now the influence of cholesterol on lipolysis, Shaw-Mackenzie [1910] concluded that cholesterol opposes the augmentation produced by sodium cholalate and it may be inferred that cholesterol acts as an inhibitor towards lipase. The influence of cholesterol was therefore investigated.

A purified cholesterol[1] was used, in the form of a coarse suspension in water, strength 0·01 M, from which the cholesterol separated on standing, and which was vigorously shaken before the requisite volumes were measured out. A 0·01 M solution in ether was also employed. In the latter case, after measuring out the requisite quantities of ethereal solution, these were added to the lipase extract, the ether was allowed to evaporate and water and emulsion were finally added. Table II *a* gives the results using a coarse aqueous suspension and Table II *b* the results employing an ethereal solution of cholesterol, in the absence of serum.

Table II *a*.

Duration of experiment, 48 hrs. Temperature 37°.

Lipase extract cc.	0·01 M cholesterol cc.	Water cc.	Emulsion cc.	$N/10$ NaOH required cc.	Effect due to cholesterol in $N/10$ NaOH cc.
1	0·0	5·0	5	24·25	—
1	0·5	4·5	5	25·30	+1·05
1	1·0	4·0	5	26·95	+2·70
1	2·0	3·0	5	26·90	+2·65
1	3·0	2·0	5	26·35	+2·10
1	5·0	0·0	5	25·25	+1·00

Table II *b*.

1	0·0	5·0	5	24·75	—
1	0·5	4·5	5	25·30	+0·55
1	1·0	4·0	5	26·10	+1·35
1	2·0	3·0	5	29·90	+5·15
1	3·0	2·0	5	29·55	+4·80
1	5·0	0·0	5	25·90	+1·15

It is clear that, so far from acting as an inhibitor, cholesterol acts as an augmentor in the concentrations employed, although, as in the case of lecithin, the effects are small compared with those produced by serum. The maximum effect in Table II *b* (5·15 cc. augmentation for 2 cc. cholesterol solution) is

[1] The writer desires to express his thanks to Dr W. A. Sexton, Dept. of Organic Chemistry, University of Liverpool, for the sample of purified cholesterol.

considerably greater than that obtained from Table II *a* (2·70 cc. augmentation for 1 cc. cholesterol suspension). The variation must be attributed to the unsatisfactory nature of the coarse suspension employed in Table II *a*.

In view of Shaw-Mackenzie's results on the influence of cholesterol on lipolysis in presence of sodium cholalate, experiments were carried out in presence of normal ox serum. The mean results of these experiments are given in Table III. The duration of experiment was 48 hours, corresponding to the maximum extent of hydrolysis observable.

Table III.

Lipase extract cc.	Ox serum cc.	0·01 *M* cholesterol cc.	Water cc.	Emulsion cc.	*N*/10 NaOH required cc.	Effect due to cholesterol in *N*/10 NaOH cc.
1	0·0	0·0	5·0	5	26·50	---
1	0·5	0·0	4·5	5	48·50	–
1	0 5	0·5	4·0	5	49·50	+1·00
1	0·5	1·0	3·5	5	48·00	0·50
1	0·5	2·0	2·5	5	47·50	1·00
1	0·5	4·5	0·0	5	46·50	2·00

The effects due to cholesterol are again small. In low concentration of cholesterol an augmentative effect is observed, changing, in higher concentrations, to an inhibitory effect. These results differ from those of Shaw-Mackenzie in that he found a strong inhibitory action by cholesterol in all concentrations. It may be pointed out that the duration of Shaw-Mackenzie's experiments from 2–18 hours gives a measure of the rate of reaction, as the writer found that 48 hours was the minimum period of reaction in order that equilibrium be established. The results of Shaw-Mackenzie and those recorded in the present paper are therefore not strictly comparable. It is evident, moreover, that cholesterol and lecithin play little part in the lipolytic co-enzymic activity of serum.

II. The influence of sodium, potassium and calcium chlorides on lipolysis.

A great deal of doubt exists as to the influence of the salts of the alkali metals and the alkaline earth metals on lipase action. Tanaka [1912] states that neutral salts of alkali metals augment lipolysis and that salts of the alkaline earths (Ca, Ba, Mg) act as inhibitors. Mellanby and Woolley [1914] state that electrolytes have no effect on the action of pancreatic steapsin. Falk [1918] concludes that sodium salts accelerate[1] but do not augment,

[1] An accelerator is here defined as a substance capable of increasing the *rate* of reaction, no account being taken of its influence on the extent of reaction. An augmentor is a substance capable of increasing the *extent* of reaction, no account being taken in this case of its influence on the rate of reaction: see footnote in previous paper by Corran and Lewis [1928]. The extent of hydrolysis observed at periods longer than 48 hours is the same as that observed at 48 hours, corresponding to a limiting extent of hydrolysis. A mass action equilibrium is, however, not assumed.

whilst calcium salts retard the rate and also diminish the extent of lipolysis. Hamsik [1911, 1915], Pekelharing [1912] and Willstätter [1923] state that sodium and calcium salts accelerate lipase action. With a view to determining the influence of the salts of the metals mentioned on the extent of lipase action, varying quantities of the salts were added to the reaction mixture and their effects investigated. The concentration of salts employed was in all cases low, 0·01 *M* aqueous solutions being used. The addition of salts was found to reduce the stability of the olive oil emulsion employed and it was found necessary to shake the reaction vessels at frequent intervals to ensure complete emulsification of the olive oil. The results are recorded in Table IV.

Table IV.

Duration of experiment, 48 hrs. Temperature 37°.

Lipase extract cc.	0·01 *M* NaCl cc.	0·01 *M* KCl cc.	0·01 *M* $CaCl_2$ cc.	H_2O cc.	Emulsion cc.	*N*/10 NaOH required cc.	Effect due to added salts in *N*/10 NaOH cc.
1	0·0	0·0	0·0	5·0	5	24·00	–
1	0·5	0·0	0·0	4·5	5	24·30	+0·30
1	1·0	0·0	0·0	4·0	5	25·90	+1·90
1	2·0	0·0	0·0	3·0	5	28·20	+4·20
1	0·0	0·5	0·0	4·5	5	24·80	+0·80
1	0·0	1·0	0·0	4·0	5	25·30	+1·30
1	0·0	2·0	0·0	3·0	5	27·80	+3·80
1	0·0	0·0	0·5	4·5	5	21·70	–2·30
1	0·0	0·0	1·0	4·0	5	19·50	–4·50
1	0·0	0·0	2·0	3·0	5	14·60	–9·40

It is evident that sodium and potassium chloride solutions oppose the action of calcium chloride. As these salts constitute the greater part of the inorganic salt constituent of serum, and moreover occur in small quantity, it is clear that the dialysable constituents of serum will have little measurable augmentative effect on lipase. The results with calcium chloride are interesting in view of Willstätter's observation that calcium chloride accelerates lipase action. The difference in reaction conditions, for example p_H of the mixture and composition of the latter, makes comparison of the results impossible.

III. The influence of copper, mercury and lead salts on lipolysis.

In an earlier paper [Corran and Lewis, 1928] results were recorded on the influence of lead, both ionic and colloidal, on the lipolytic augmentation produced by serum. In every case increases in the effects due to serum were recorded. More recently the influence of various metallic salts has been investigated, generally in the absence of serum, but in some cases also in the presence of serum. 0·01 *M* aqueous solutions of cupric and mercuric chlorides and 0·01 *M* lead acetate were used. In all cases the duration of experiment was 48 hours and the temperature 37°.

Table V. *Influence of* 0·01 *M lead acetate on lipase in absence of serum.*

Lipase extract cc.	0·01 *M* Pb acetate cc.	Water cc.	Emulsion cc.	$N/10$ NaOH required cc.	Augmentation in $N/10$ NaOH cc.
1	0·00	5·00	5	25·70	—
1	0·25	4·75	5	26·10	+ 0·40
1	0·50	4·50	5	27·70	+ 2·00
1	1·00	4·00	5	30·90	+ 5·20
1	2·00	3·00	5	38·10	+ 12·40
1	5·00	0·00	5	40·10	+ 14·40

Table VI. *Influence of* 0·01 *M cupric chloride on lipase in absence of serum.*

Lipase extract cc.	0·01 *M* $CuCl_2$ cc.	Water cc.	Emulsion cc.	$N/10$ NaOH required cc.	Augmentation in $N/10$ NaOH cc.
1	0·00	5·00	5	22·50	—
1	0·50	4·50	5	20·60	− 1·90
1	1·00	4·00	5	18·55	− 3·95
1	2·00	3·00	5	14·00	− 8·50
1	3·00	2·00	5	6·70	− 15·80
1	5·00	0·00	5	2·60	− 19·90

Table VII. *Influence of* 0·01 *M cupric chloride in presence of serum.*

Lipase extract cc.	Ox serum cc.	0·01 *M* $CuCl_2$ cc.	Water cc.	Emulsion cc.	$N/10$ NaOH required cc.	Effect due to $CuCl_2$ in $N/10$ NaOH cc.
1	0·0	0·00	5·00	5	22·00	
1	0·5	0·00	4·50	5	44·00	
1	0·5	0·25	4·25	5	41·10	− 2·90
1	0·5	0·50	4·00	5	34·60	− 9·40
1	0·5	1·00	3·50	5	29·50	− 14·50
1	0·5	2·00	2·50	5	26·50	− 17·50
1	0·5	4·50	0·00	5	6·50	− 37·50

Table VIII. *Influence of* 0·01 *M mercuric chloride on lipase in presence of serum.*

Lipase extract cc.	Ox serum cc.	0·01 *M* $HgCl_2$ cc.	Water cc.	Emulsion cc.	$N/10$ NaOH required cc.	Effect due to $HgCl_2$ in $N/10$ NaOH cc.
1	0·0	0·00	5·0	5	21·50	
1	0·5	0·00	4·5	5	43·17	
1	0·5	0·50	4·0	5	40·00	− 3·17
1	0·5	1·00	3·5	5	37·25	− 5·92
1	0·5	2·00	2·5	5	34·15	− 9·02
1	0·5	4·50	0·0	5	8·33	− 34·84

Copper exerts an inhibitory action on lipase in absence of serum. Moreover, both copper and mercury exert a similar effect on the co-enzymic activity of serum. On the other hand, lead exerts an augmentative effect on lipase, both in presence and in absence of serum. It might be of advantage to compare the augmentations produced by lead acetate in absence and in presence of serum. In absence of serum, 0·5 cc. of 0·01 *M* lead acetate solution gives an augmentation (in terms of $N/10$ NaOH) of 2·00 cc. In presence of serum[1] a similar quantity gives rise to an augmentation of 7·12 cc. Whether lead exerts an influence on the enzyme alone or whether it interacts, in addition, with the co-enzyme in serum, is not clear.

[1] These values are the minimum values recorded in an earlier paper [Corran and Lewis, 1928].

IV. The lipolytic co-enzyme in serum.

With the object of endeavouring to ascertain to which of the various constituents of serum the lipolytic co-enzyme may be attributed, and, if possible, of isolating the co-enzyme itself, ox-serum was submitted to a separation into its constituents.

Fresh ox-serum, previously centrifuged to remove any red blood-corpuscles, was employed. 50 cc. were dialysed in a collodion membrane, the distilled water in the surrounding vessel being changed every 6 hours. During dialysis, a precipitate was formed inside the membrane due to precipitation of a portion of the proteins in the serum. The dialysates were collected and thus the dialysable inorganic constituents of serum obtained. The mixture inside the membrane, consisting of serum freed from dialysable salts, was then filtered, the residue inside the filter-paper consisting of the euglobulin fraction. The filtrate was then acidified with a few drops of acetic acid and heated in a water-bath at 100°, when the proteins contained therein were precipitated. Thus the albumin + pseudoglobulin fraction was obtained, leaving in solution, after filtration, the lipins and their derivatives. No further separation was carried out. The fractions isolated, namely, inorganic compounds, euglobulin, pseudoglobulin + albumin, and lipins, were then made up to 50 cc. with water or, alternatively, evaporated to the required volume, *i.e.* so that the concentration of each constituent was identical with its concentration in the original serum, the protein fractions being in the form of coarse suspensions, which were shaken vigorously before volumes were measured out. Samples of 0·5 cc. of each mixture were taken and their lipolytic augmentations determined in the manner previously described. The accompanying table gives the results obtained.

Table IX.

Lipase extract cc.	Inorganic salts cc.	Euglobulin fraction cc.	Albumin + pseudo-globulin fraction cc.	Lipin fraction cc.	H_2O cc.	Emulsion cc.	$N/10$ NaOH required cc.
1	0·0	0·0	0·0	0·0	5·0	5	24·00
1	0·5	0·0	0·0	0·0	4·5	5	24·00
1	0·5	0·0	0·0	0·0	4·5	5	23·80
1	0·0	0·5	0·0	0·0	4·5	5	27·80
1	0·0	0·5	0·0	0·0	4·5	5	28·00
1	0·0	0·0	0·5	0·0	4·5	5	40·70
1	0·0	0·0	0·5	0·0	4·5	5	40·90
1	0·0	0·0	0·0	0·5	4·5	5	24·10
1	0·0	0·0	0·0	0·5	4·5	5	24·20

Table X. *Mean augmentations produced by each fraction of serum.*

	cc. $N/10$ NaOH
Inorganic salts	0·00
Euglobulin fraction	3·90
Albumin + pseudoglobulin fraction	16·80
Lipin fraction	0·15
Total	20·65 cc. $N/10$ NaOH

(0·5 cc. of untreated fresh ox-serum gives an augmentation of 22·0 cc. $N/10$ NaOH.) It is noteworthy that the total effects are as close together as they are in view of the somewhat drastic alterations in the physico-chemical state of the protein fractions.

It is evident that the albumin + pseudoglobulin fraction contains the greater part of the lipolytic co-enzyme. The value due to the euglobulin fraction may be due to the co-enzyme present in this fraction or possibly to incomplete separation of the albumin and euglobulin fractions. It is interesting to note that the inorganic salt fraction and the lipin fraction have no co-enzymic effect, thus bearing out the results recorded in a previous section of this paper.

Summary.

(1) The influence of a number of different compounds on lipolysis has been investigated.

(2) The quantitative effects of purified lecithin and cholesterol were similar and only slight. In low concentration both compounds act as augmentors, the values of the augmentations subsequently falling with increasing concentration.

(3) The influences of sodium and potassium chlorides on lipase are only slightly augmentative. Calcium chloride, on the other hand, acts as an inhibitor, its quantitative effect being greater than that of sodium or potassium chloride.

(4) Ionic lead acts as an augmentor towards lipase in the presence and in the absence of serum. Copper, both in absence and in presence of serum, acts as an inhibitor, as does mercury.

(5) The albumin + pseudoglobulin fraction of blood-serum was found to possess over 80 % of the co-enzymic activity exhibited by serum.

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XXVI. THE NATURE OF THE PECTIC SUBSTANCES OF FLAX.

A PRELIMINARY INVESTIGATION.

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As the result of recent researches our knowledge of the pectic substances of plants has become more systematic, and it is proposed in the present paper to adhere to the nomenclature adopted by the American Pectin Symposium of 1925. Thus we are now acquainted with protopectin, the naturally occurring pectic substance; pectin, the substance extracted from the plant by chemical or other means with the minimum of chemical degradation; and pectic acid, a derived product.

For the purposes of the present communication it is necessary to refer briefly to some recent investigations, commencing with the isolation of pectic acid [Clayson, Norris and Schryver, 1921]. It was shown that pectic acid could be prepared from a large variety of plant sources, that it appeared to be of constant, or very nearly constant, composition without reference to the particular source, and was, in fact, a definite chemical entity. Important researches, notably by Ehrlich [1917, 1924], culminated in the suggestion of a ring formula for pectic acid by Nanji, Paton and Ling [1925]. According to this suggestion, pectic acid is the basal substance to which the other pectic substances may be referred, and the molecule is a closed six-membered ring, whose members represent one molecule of anhydro-arabinose, one molecule of anhydro-galactose and four molecules of galacturonic anhydride, the four carboxyl groups of the last being free. Pectin, or the "pectinogen" of Schryver and Haynes [1916] and Norris and Schryver [1925], contains, in addition, methyl alcohol to a varying extent, in ester combination with the free carboxyl groups of the original pectic acid.

No serious objections to this suggested formula have been advanced since its publication, nor has the uniform nature of pectic acid derived from different sources been disputed. Only in one case has there been any indication of a departure from this generalisation, that of the pectic substance of flax. Ehrlich and Schubert [1926] suggested the presence of xylose in addition to the usual constituents of the molecule, but this has not been confirmed. In a recent paper on the subject, Henderson [1928] casts some doubt on the validity of the ring formula in the case of flax pectin, and rejects it in favour of a simpler galactose-tetragalacturonic acid formula:

$$(C_6H_{10}O_5 . 4C_6H_8O_6 - H_2O)_n;$$

arabinose is regarded as an adventitious adsorbed impurity.

The present investigation was undertaken to test the correctness of Henderson's criticisms. His method of work was not repeated exactly, as it was considered advisable at the present stage to prepare the pectic substances by the general methods which have been adopted in previous communications [Clayson, Norris and Schryver, 1921; Norris and Schryver, 1925]. Thus there is nothing novel in the methods adopted, which consisted of a first treatment of unretted flax with hot water until colourless washings were obtained. The flax was then divided into two portions, each representing 200 g. of the original flax. One portion was treated directly with 0·5 % ammonium oxalate solution at 95–98° for 2 hours; the extract was then filtered off and precipitated with 2 volumes of 95 % alcohol. The gel was purified by re-solution in distilled water and reprecipitation in alcohol some five or six times, when a colourless product was finally obtained. This is the soluble pectin, the "pectinogen" of Schryver and the "ammonium oxalate pectin" of Henderson.

The other portion of flax was treated first with cold 4 % sodium hydroxide, which was subsequently completely washed from the fibre. The latter was then treated as usual with hot 0·5 % ammonium oxalate solution, and a gel was precipitated from this extract by concentrated hydrochloric acid. The gel in this case was not free from colour, but after repeated solution in the absolute minimum of dilute ammonia, and reprecipitation in acid alcohol, most of the colour was removed and a good sample of pectic acid obtained.

The ash content, furfural and carbon dioxide produced on hydrolysis with 12 % hydrochloric acid were determined for each product, and in the case of the soluble pectin, methoxyl groups were also estimated, the results being shown below.

	Pectic acid %	Pectin %
Ash	0·99	5·3
Furfural	20·16 (average of 3 det.)	20·9 (average of 3 det.)
Carbon dioxide	17·80 (average of 3 det.)	Not det. owing to lack of material
Methoxyl	—	9·65 (average of 4 det.)

Discussion of results.

The results summarised above are quite in accordance with expectations based on the ring formula of Nanji, Paton and Ling. Considered side by side with analyses of pectic acid and pectin from widely different sources, they present no unusual features. This is clearly indicated by reference to Table I (Nos. 17 and 18).

Yield of carbon dioxide on hydrolysis.

Henderson bases his suggested formula on this value, which he finds to be an increasing one with increasing apparent purity of the sample. Thus, on repeated re-solution in caustic soda, followed by reprecipitation with acid alcohol, a product is finally obtained which yields 20·9 % of carbon dioxide. Although most of the experimental errors in the determination of carbon

dioxide are likely to lead to high results, this progressive increase is difficult to understand, especially in view of the fact that recent work by Candlin and Schryver [1928] would seem to indicate that a loss of radicles which yield carbon dioxide takes place on prolonged treatment of pectin with caustic soda.

Table I. *Analyses of pectic substances.*

	Nature of product	Source	Reference	Furfural %	CO_2 %
1.	Pure pectic acid	Pea-pod	Clayson, Norris and Schryver [1921]	20·7	—
2.	,,	Turnip	,, ,,	21·6	—
3.	,,	Onion	,, ,,	20·7	—
4.	,,	Orange	,, ,,	21·5	—
5.	,,	Cabbage	,, ,,	20·3	—
6.	,,	Apple	,, ,,	21·7	—
7.	Crude pectin	,,	Nanji, Paton and Ling [1925]	—	13·79
8.	Purified pectin	,,	,, ,,	—	18·56
9.	Pectin A	Beet	,, ,,	—	18·08
10.	,, B	,,	,, ,,	—	19·81
11.	Pectic acid	Orange	,, ,,	—	18·09
12.	,,	Onion	,, ,,	—	18·00
13.	Calcium pectate (calc. on ash-free basis)		,, ,,	—	17·64
14.	Pectic acid	Orange juice	Norris [1926]	20·4	—
15.	Pectin	,,	,,	19·2	—
	Average of observed figures			20·75	17·71
	Calculated figures from ring formula			20·85	17·64
	Calculated from Henderson formula			14·03	20·70
16.	Pectic acid	Flax	Henderson [1928]	—	20·90
17.	,,	,,	Present communication	20·16	17·80
18.	Pectin	,,	,,	20·90	—

In discussing the results of Nanji, Paton and Ling. Henderson suggests that the yields of carbon dioxide obtained by them approach the higher figure, 20·7 %, required by his formula, and that with further purification of the products this figure might have been reached. In point of actual fact, the reverse is true—with increasing purity of product, their results more nearly approach the lower figure, 17·6 %, required by the ring formula. These results are shown in the table (Nos. 7–13); calcium pectate (No. 13) can be prepared in a high state of purity, and, with a carbon dioxide yield of 17·64 % (on ash-free basis), gives the exact figure required by the ring formula. Of the other products, the pectic acids, Nos. 11 and 12, were very carefully prepared and purified by the writer and give results of 18·09 and 18·00, again very nearly approaching the theoretical figure required by the ring formula. Pectic acid obtained from flax in the present instance (No. 17) shows a result, 17·8 %, much more closely in agreement with that required by the ring formula than with the formula of Henderson.

Yield of furfural on hydrolysis.

The case for the formula suggested by Henderson is not strengthened by his omission to quote the yields of furfural obtained from his products. The average yield of furfural from pectin is about 20·75 % (see Table I); the theoretical yield on the basis of the ring formula is 20·85 %, and in the present instance the pectic acid and pectin from flax give 20·16 and 20·9 % respectively.

These figures are in good agreement, therefore, with the [illegible]ly obtained, and here it may be remarked that the calculated figures are only approximate, although the errors involved in the calculation are of the same order as the experimental ones.

If the suggestion of Henderson, that arabinose is an adsorbed and consequently variable impurity, is correct, then no constant value for furfural could be expected. Further, a substance of the type suggested by him—galactose-tetragalacturonic acid—would yield only approximately 14 % of furfural. It is extremely unlikely that so low a figure has ever been quoted in the literature in the case of a pure pectic substance, and it is quite outside the range indicated in the table. The apparent constancy of the furfural yield—*circa* 20 %—militates against the suggestion that arabinose is a variable constituent of pectin.

It may be remarked in passing that the methoxyl content, 9·65 %, is also quite a normal figure for pectin produced by 2 hours' extraction with hot 0·5 % ammonium oxalate.

It is possible that the abnormal results obtained by Henderson may be due to an impurity, in spite of protracted attempts at purification. Thus it is highly probable that pectin is frequently contaminated with substances of the type known as hemicelluloses [Norris and Schryver, 1925]; further, O'Dwyer [1928] has indicated that this type of substance contains methoxyl in a form of combination which renders it highly resistant to the action of alkali. The observation by Henderson, that even after purification his product still contained a small quantity of residual methoxyl, is in harmony with the suggestion that hemicellulose might be an impurity.

In conclusion it cannot be said that a valid argument has been advanced by Henderson, and there seems to be no adequate reason for rejecting the ring formula in the case of the pectic substances of flax.

The results obtained by Henderson with reference to the progressive hydrolytic breakdown of pectin are of considerable interest and will form the subject of a further communication.

The author desires to express his thanks to Prof. A. R. Ling for his interest in the present work, and to Mr A. C. Hulme for practical assistance.

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XXVII. STUDIES IN THE METABOLISM OF TISSUES GROWING *IN VITRO*.

III. CYANIC ACID AS A POSSIBLE PRECURSOR OF THE AMMONIA AND UREA FORMED BY EMBRYO KIDNEY TISSUE.

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(*Received February 28th, 1929.*)

It has already been shown [Holmes and Watchorn, 1927; Watchorn and Holmes, 1927] that embryonic kidney tissue of the rat growing *in vitro* is capable of forming both ammonia and urea, while under the same conditions non-growing tissue is inactive in this respect. For the sake of clearness, it is necessary to recapitulate this work to some extent, but for details earlier papers should be consulted. Ammonia and urea are estimated by a modification of Stanford's [1923] method, as previously described. The medium employed is an embryonic tissue extract, made up in Pannett and Compton's [1924] modification of Ringer's solution. The controls used are the following:

(A) 2 cc. of medium, kept at 0° for 48 hours;

(B) 2 cc. of medium, plus the same amount of embryo kidney tissue as is used in the experimental flasks; this is also kept at 0° for 48 hours:

(C) 2 cc. of medium incubated for 48 hours.

The experimental preparations are also incubated for 48 hours and consist of 2 cc. of medium plus embryo kidney tissue. From control B the amounts of ammonia and urea in the medium and kidney tissue at the beginning of the experiment can be determined, while C − A (the difference is usually small) allows for any ammonia and urea which may be formed by the medium itself during the 48 hours' incubation through autolytic or other processes. The total control is thus represented by B + (C − A). If the content of ammonia and urea in the experimental preparations is in excess of that in the total control, it is plain that the extra amounts have been formed by the activity of the tissue explants.

The growing tissue is supported upon cotton wool strands, while growth is prevented in the "resting" or non-growing preparations by lack of mechanical support. The depth of fluid even in this case is very small, but is just sufficient to float the minute fragments of tissue.

We have always found that the content of urea- and ammonia-nitrogen in the non-growing specimens is the same as that of the total control, that is to say these substances are not formed, under our conditions, by non-growing tissue. On the other hand the growing tissues form very considerable amounts

of both these substances. Quite early in the course of the work it became obvious that sometimes it was chiefly ammonia that was formed, and sometimes chiefly urea, although there was no reason to suppose that the conditions could have varied from one experiment to another. Moreover we have sometimes found that when one experimental series included two growing preparations, the total rise in ammonia- and urea-nitrogen might be the same in both of them, but in one case it might be predominantly urea and in the other predominantly ammonia formation which accounted for the rise. The medium on the other hand would be identical in the two cases, and the kidneys taken from embryos of the same litter, so that conditions would not differ to any appreciable extent. The following example is taken from an experiment giving this type of result:

	Total control mg.	1st grower mg.	2nd grower mg.
NH_3-N	0·060	0·073	0·084
Urea-N	0·027	0·054	0·040
NH_3 + Urea-N	0·087	0·127	0·124

It is quite certain that rat kidney tissue does not contain urease, so that in no case can the ammonia be formed from urea, and, on the other hand, we have never obtained any results suggesting that the tissue is capable of converting ammonia into urea. In order to test this point we have carried out experiments in which ammonia has been added to the medium. In no case, either in floating or growing preparations, has there been any utilisation of the ammonia to form urea.

It seems therefore, that we cannot account for the apparently alternative appearance of urea and ammonia during growth by assuming that either of these substances, once formed, can be converted into the other.

The simplest explanation of the results is that there is a common precursor of urea and ammonia, which may be easily converted into either of these, and which is made by the growing tissues during the breakdown of nitrogenous substances.

Werner [1923] and Fearon and Montgomery [1924] have suggested that cyanic acid may arise in the animal body as a result of the deamination of amino-acids, and that this could then give rise (as it is, of course, well known to do *in vitro*) to both ammonia and urea. Fearon and Montgomery have shown that cyanate may be formed during the oxidation of amino-acids *in vitro* (particularly in the presence of carbon compounds), but the evidence for its formation in the animal body is very slender.

However, if Werner's theories are correct, we may imagine that the growing preparations of kidney tissue can form cyanic acid during the deamination of the protein derivatives contained in the medium. The non-growing tissues do not carry out these deaminations to any extent, but if supplied with cyanic acid from outside they should produce ammonia and urea from it, so that a non-growing preparation to which cyanic acid had been

added might give the same type of result as a growing preparation with an ordinary medium. A series of experiments was undertaken to test this point.

Neutralised potassium cyanate (p_H 7·2), which contains, of course, a considerable proportion of free cyanic acid, was used. The Ringer's solution containing cyanate was sterilised by filtration through a candle, in order to avoid breakdown of the cyanate. The final concentration was such that about 0·05 or 0·06 mg. of cyanate-nitrogen was present in 2 cc. medium. The best results are obtained with embryos two or three days before their expected birth. It was very necessary to show that the cyanate in this concentration was not toxic to the tissues, as dead tissue would probably give rise to ammonia and urea by autolysis. Many microscopical examinations were therefore carried out, and it is safe to say that the cyanate did not inhibit the growth of embryonic kidney tissue and it is very improbable that it was appreciably toxic at this dilution. The objection of the extreme toxicity of cyanate has often been brought forward against the Werner and Fearon theory. This objection is plainly not valid, and it must be pointed out that the concentration of cyanic acid in our medium (7·5-9·0 mg. cyanic acid per 100 cc.) is considerably greater than that supposed by Montgomery [1925] to be present in blood.

Table I.

	Ammonia-N		Urea-N		Total urea-N + NH_3-N	
Exp. No.	Control mg.	Resting tissue mg.	Control mg.	Resting tissue mg.	Control mg.	Resting tissue mg.
54	0·034	0·045	0·016	0·016	0·050	0·061
59	0·026	0·021	0·021	0·052	0·050	0·073
60	0·036	0·054*	0·040	—	0 076	—
		0·050		0·048		0·098
66	0·030	0·048	0·016	0·021	0·046	0·069
		0·043		0·019		0·062
69	0·026	0·039	0·028	0·028	0·054	0·067
		0·033		0·050		0·083
70	0·034	0·035	0·032	0·050	0·066	0·085
		0·048		0 065		0·113
72	0·028	0·054	0·042	0·044	0·070	0·098
		0·060		—		—

* Where two sets of figures are given, these refer to two separate preparations in the same experimental series, the control therefore being the same for both.

The results are given in Table I, and it can be seen that in the presence of non-growing embryonic kidney tissue the added cyanate is partly broken down, yielding ammonia or urea, or a mixture of the two, thus giving results exactly like those found in the case of growing tissue without added cyanate. When no tissue was present, and the medium incubated by itself (control C), there was not usually any perceptible breakdown of the cyanate, though this did occur on one or two occasions (these experiments are not quoted in the table). It is therefore undoubtedly true that the kidney tissue can catalyse the breakdown of the cyanate in some way, though whether by direct enzymic action or by indirect physical means, such as local alterations of p_H in the medium, it is not possible to say.

No breakdown of cyanate occurs during the course of the estimations, provided that the temperature is not raised above 55° during the distillations.

Although these experiments cannot be held to prove that cyanic acid is formed by the nitrogenous metabolism of growing tissues, it can be said that if cyanic acid is formed during growth then the results which we have formerly described can be very satisfactorily explained.

Werner [1923] suggests that the following equation represents the formation of urea from cyanate:

$$2HN{:}CO + H_2O = CON_2H_4 + CO_2$$

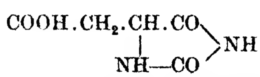
whereas in the presence of extra ammonia he thinks that more urea would be formed from the same amount of cyanate, the ammonia being utilised:

$$2HN{:}CO + 2NH_3 = 2CON_2H_4.$$

According to this theory, the tissues growing in embryo extract, if they were in fact forming cyanate, should utilise some of the ammonia already present to form urea. This, however, never occurred, even when extra ammonia was added to the medium. This does not show that cyanate is not formed during growth, as we were at first inclined to think, since when cyanate was actually added to the medium there was no sign of ammonia utilisation, nor was there anything to suggest that the proportion of urea formed depended upon the amount of ammonia present. Possibly the concentrations of ammonia used (about 3 mg. per 100 cc.) were not sufficient to affect the reaction. During life there may be local concentrations far greater than this, particularly in the functioning kidney and liver, but larger amounts, if added to the medium of cultures, would probably prevent growth.

The breakdown of 1: *hydantoinacetic acid.*

Some time ago Dakin [1908] described the appearance in the urine of the uramido-acid from phenylalanine when the latter was injected intravenously into a rabbit, and also of both the uramido- and the hydantoin derivatives when inactive tyrosine was fed to cats [Dakin, 1910, 1]. He suggested that urea might be formed directly from these without the intermediate formation of ammonia. He later [1926] pointed out that if cyanic acid were present in the body, it would probably react with amino-acids to give uramido-acids and hydantoin. When the uramido- and hydantoin derivatives of *dl*-phenylalanine and *dl*-leucine were fed to rabbits, they were excreted unchanged. However, when the derivatives of *d*-glutamic acid were fed, only a small proportion could be recovered, and the hydantoinacetic acid from *l*-aspartic acid was also mainly destroyed in the body.



Hydantoinacetic acid

It seems probable from Dakin's work that these substances are normally formed during metabolism and as their fate is of biochemical interest in connection with the cyanate theory of urea formation in the body, we prepared *l*-hydantoinacetic acid from *l*-aspartic acid and potassium cyanate according to the directions given by Dakin [1910, 2]. The hydantoin was then added to the medium in sufficient amounts to bring the final concentration to 0·06–0·08 %, and the Ringer's solution containing the hydantoin was sterilised by steaming three times. A few experiments only have been carried out, but they show quite definitely that the embryo kidney tissue can break down the hydantoin and form urea from it without necessarily showing any formation of extra ammonia. This breakdown may also occur in the medium when this is incubated by itself: the enzymes concerned are therefore probably not peculiar to the kidney, but can be extracted from other embryonic tissues. For instance, in one experimental series the urea-N of the total control should have been, judging by the controls kept at 0°, about 0·02 mg. whereas the urea-N in the incubated medium had increased to 0·03 mg. in one preparation and 0·054 mg. in another. A non-growing tissue preparation in the same series contained 0·043 mg. urea-nitrogen. No extra ammonia was found. In one experiment ammonia was produced, and not urea, the amounts being as follows: ammonia-N in total control 0·044 mg., and in two non-growing preparations 0·057 and 0·061 mg. respectively. It is therefore possible that cyanic acid and not urea itself can be first split off from the hydantoin, and give rise to either urea or ammonia.

It is not possible to say from these results whether hydantoin formation plays an important part in the production of urea from cyanate by kidney tissue.

Utilisation of urea by embryonic kidney tissue.

During the course of experiments with added cyanate we found that the growing preparations often showed a very considerable fall in urea content (Table II). It is not at all probable that this reaction would complicate the formation of urea from cyanic acid, supposing the latter to be formed during growth. It is reasonable to suppose that cyanic acid, if formed, would be broken down quickly, and would never accumulate to any extent. In this case the urea formation from cyanic acid would be the predominant reaction, and not the disappearance of urea that is noticeable when considerable amounts of cyanate have been added.

Table II.

Exp. No.	Total control Urea-N mg.	Non-growing tissue Urea-N mg.	Growing tissue Urea-N mg.
54	0·024	—	0·014
57	0·028	—	0·016
60	0·040	0·031	—
62	0·026	0·011	—
65	0·050	0·022	0·008
68	0·073	—	0·050
72	0·038	—	0·026

N.B. In all the above experiments cyanate was added to the medium.

The fall in urea content may be quite large, and when the fact that urea production is probably proceeding at the same time is taken into consideration, it will be seen that the amounts of urea utilised are very considerable. We know that the urea is not broken down to ammonia, so that it is probably taking part in a synthesis; as yet, however, we have no further knowledge of its fate.

Some of the examples given in Table II show a fall of urea-nitrogen in the non-growing tissue as well as in the growing. In the former it is more usual, as already pointed out, to find a rise in urea due to the breakdown of the cyanate, and the loss of urea is always greater when growth occurs. The incubated medium (control C) often showed a very slight decrease in its urea content when compared with the ice-chest medium (control A). That this decrease is real, and not an experimental error, is shown by the fact that on one occasion the drop in urea-N was considerable (over 0·01 mg.). Evidently then, the disappearance of urea does not entirely depend upon growth, or even upon the presence of embryo kidney tissue, but can be brought about to some extent by the embryo extract used for the medium.

The disappearance of urea during growth had previously been noticed [Watchorn and Holmes, 1927] when the medium contained additional glucose, though never when the plain embryo extract was used.

Summary.

(1) The possibility that cyanic acid may be the precursor of ammonia and urea formed by growing kidney tissue has been discussed, and experiments have been described to test this point.

(2) Cyanic acid in the presence of embryo kidney tissue is broken down to ammonia and urea, and the tissue catalyses the reaction.

(3) Cyanic acid does not appear to have a toxic effect upon the tissue, when it is present in amounts up to 9·0 mg. per 100 cc. of medium.

(4) *l*-Hydantoinacetic acid, which might arise in the body as the result of a reaction between *l*-aspartic acid and cyanic acid, is also broken down by embryonic tissue, and gives rise to urea and ammonia.

(5) In the presence of cyanate, urea may disappear from the cultures. This is particularly the case when the tissue is actively growing.

Our best thanks are due to Miss A. Patey, who prepared for us a specimen of pure potassium cyanate, and who also on one or two occasions very kindly helped us in the technique of setting up the cultures.

We are indebted to the Medical Research Council for grants which have made this work possible.

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XXVIII. THE EFFECT OF EXCESSIVE DOSES OF IRRADIATED ERGOSTEROL ON THE CALCIUM AND PHOSPHORUS CONTENT OF THE BLOOD.

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(*Report to the Medical Research Council.*)

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In earlier papers of this series [Harris and Moore, 1928, 1, 2, 1929], a description has been given of some of the effects produced by large overdoses of vitamin D (irradiated ergosterol). Amongst these effects is the extensive deposition of calcium salts in various parts of the body. This finding, together with the well known effects of deprivation of vitamin D, rendered it desirable to investigate the effect of overdosage of the vitamin on the calcium and phosphorus content of the blood. Hess and Lewis [1928] indeed, had noticed several cases of hypercalcaemia and three cases of abnormally high blood-phosphorus in a series of rachitic patients treated with irradiated ergosterol at the rate of 2·5 to 5 mg. *per diem*.

This note is intended to give a preliminary report of our results. Publication at the present juncture is prompted by the appearance of a note by Hess, Weinstock and Rivkin [1928] which has just come to our notice. These authors found that in young rats, on a diet high in phosphorus but containing negligible amounts of calcium, so that the serum-calcium was very low (6·4 mg. per 100 cc.), the serum-calcium was rapidly raised towards a more normal level by daily administration of 1 mg. of irradiated ergosterol. Our own experiments differ from those of Hess and his co-workers in two important particulars—we used adult animals, and made no attempt to restrict the calcium intake below the normal.

The main experiment we have to describe was carried out with adult rabbits which were allowed an unlimited diet of oats and cabbage leaves. Water was offered to the animals, but was never touched. Six rabbits were used and, after a control period during which samples of blood were withdrawn periodically for estimation of the serum-calcium by the method of Kramer and Tisdall [1921] and of the inorganic phosphate of the blood by the method of Briggs [1924], were divided into three groups. The first group received daily 0·5 cc. of arachis oil; the second received 0·5 cc. arachis oil containing 10 mg. non-irradiated ergosterol; the third received 0·5 cc. of arachis oil containing 10 mg. irradiated ergosterol[1]. The oil was administered

[1] The ergosterol was irradiated in alcohol as previously described [Harris and Moore, 1928, 1, 2].

by pipette, lest mixing with the ordinary food should lead to its refusal. The rabbits proved unexpectedly resistant to overdosage of the vitamin, and it was only after nearly a month of this treatment that any change in the blood-Ca or -P was noted. Although no record was kept of the food consumption it was noted, 25 days after the first dose, that the two rabbits receiving irradiated ergosterol had consumed much less than usual. The following day food was again refused, and on that day the blood showed an increased content of inorganic phosphorus, the serum-calcium, however, remaining normal. A fortnight later the blood-phosphorus was still high, about 50 % above normal, and the serum-calcium showed a slight increase. The control animals showed no significant change throughout. (The figures are given in Table I.)

Table I.

P = mg. inorganic phosphorus per 100 cc. blood.
Ca = mg. calcium per 100 cc. serum.

	No ergosterol				Non-irradiated ergosterol				Irradiated ergosterol			
	I		II		III		IV		V		VI	
Date	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca
Dec. 10th	4·20	10·9	4·50	11·7	4·50	9·7	4·60	12·0	5·00	11·4	5·00	11·8
,, 15th	4·45	11·0	4·60	11·4	4·30	9·5	4·00	11·0	4·50	12·4	5·40	11·6
,, 20th	4·00	10·4	4·30	10·3	4·70	10·3	4·50	11·5	5·30	13·0	5·10	12·0
,, 31st	4·12	10·7	4·40	10·9	4·10	10·0	4·00	11·8	5·70	11·8	5·30	11·5
Jan. 3rd	4·30	10·3	4·35	10·4	4·30	10·5	4·40	11·3	5·40	12·0	5·00	11·4
	End of control period. Feeding started											
,, 7th	4·35	10·6	4·28	10·3	4·00	10·2	4·30	11·0	5·10	11·8	5·20	11·7
,, 10th	4·00	10·7	4·12	10·5	4·44	10·3	4·00	11·4	5·72	11·8	5·00	12·0
,, 14th	4·20	11·0	4·40	10·0	4·40	9·9	4·20	10·8	4·70	11·7	5·30	11·8
,, 31st	4·41	11·3	4·17	10·6	5·00	--	4·30	11·1	7·50	11·6	7·50	11·8
Feb. 13th	4·10	11·5	4·35	11·3	4·70	10·1	4·15	11·5	7·65	12·5	8·00	12·6

We hesitate to accept the last estimations on rabbits V and VI as showing a definite rise in the serum-calcium. Certainly they are higher than the preceding ones, but the normal fluctuations in the serum-calcium of rabbits are often as great as the difference between the last and penultimate analyses. Unfortunately at the time we were in ignorance of Hess's work on the administration of vitamin D to rats, and the rabbits were killed at this stage and examined *post mortem* for the characteristic changes.

Post mortem examination showed that the usual deposition of calcium salts had taken place. That in the kidney was visible to the naked eye, and the urine in the bladder was noticeably cloudy and found to be saturated with calcium salts. The rabbits had not lost weight, however, and, though eating less than normally, were by no means starving.

The absence of a definite hypercalcaemia, even if it be admitted that the approaching onset of such a condition is indicated, may be due to the relatively high resistance of the adult animals to overdosage with vitamin D and adequate excretion. A larger dose, or continuation of the experiment for a longer period, might have produced a definite increase in the serum-calcium.

It seems unlikely that the larger amounts of calcium fed to our animals is the cause of the difference between our results and those reported by Hess in cases of experimental hypocalcaemia. In this connection it is interesting to note that Hess, Weinstock and Rivkin [1928] were unable to bring about, in adult rats, the rapid fall in the serum-calcium which, in young animals, was produced by the low calcium-high phosphorus diet which they employed. At the same time, the composition of the diet cannot be ignored as a possible factor in determining the precise effect of overdosage of vitamin D. Deficiency of the vitamin may be followed by a low blood-phosphorus or a low blood-calcium, or both, with of course faulty calcification, according to the diet; it is quite possible that excess of the vitamin may cause high phosphorus or high calcium, or both, again according to the diet. Hess states that he is investigating this point.

In any case, the degree of hypervitaminosis must have some part in deciding the blood-picture, and in an experiment on young rats (100 g.) to which irradiated ergosterol (0·1 % of diet) was administered until, after 14 days, a loss of from 20 to 30 g. in weight had resulted, we have found again a high inorganic phosphorus content of the blood and also a definitely raised serum-calcium. The blood, a mixed sample from two rats, contained 7·0 mg. of inorganic phosphorus per 100 cc., an amount about 50 % above the normal, while the mixed serum contained 12·6 mg. of calcium per 100 cc., about 25 % more than the normal, which in rats is about 10 mg., with a range of 9·5 to 10·5. The animals used in this experiment received a diet containing an adequate amount of calcium and phosphorus in normal ratio.

Further experiments are being carried out to show the effect on the calcium and phosphate content of the blood of different degrees of hypervitaminosis, and an attempt is being made to investigate the significance of the increase in inorganic phosphorus which, according to the preliminary experiments here described, seems to precede the hypercalcaemia.

Summary.

1. In the young rat, the administration of a complete synthetic diet (normal Ca-P ratio) containing 0·1 % of irradiated ergosterol was followed by a 50 % increase of blood-inorganic phosphate and a 25 % increase of serum-calcium.

2. In adult rabbits receiving a normal mixed diet, administration of 10 mg. *per diem* of irradiated ergosterol per animal was followed, after upwards of a fortnight at the normal level, by a 50 % increase in the blood-inorganic phosphate. After six weeks there was still no significant rise in serum-calcium although abnormal deposits of calcium had already appeared in the body.

The authors' thanks are due to Dr A. C. White, of Edinburgh University, who undertook the care of the rabbits used in this work, and who administered the doses of vitamin and obtained the samples of blood for analysis.

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Vol. 23, p. 209: *for* Harris and Moore (1929). *Biochem. J.* **23**, 206.
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XXIX. GLYCOLYSIS IN MUSCLE AND OTHER TISSUES.

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(*Received March 2nd, 1929.*)

As has been pointed out by Meyerhof [1927], the rate at which glucose is utilised by the mammalian muscle enzyme system is very much smaller than that at which glycogen is broken down to lactic acid. This is true whether minced muscle or muscle extract be employed, but in the latter case the difference is much more marked; in fact it is commonly found that an extract highly active for glycogen is practically without action upon glucose in similar concentration. In presence, however, of an activator whose preparation from yeast is described by Meyerhof, such an extract acquires the power of dealing with glucose and other fermentable hexoses.

It is well known that certain tissues other than muscle, *e.g.* brain and kidney, habitually metabolise glucose with formation of lactic acid; this has been demonstrated in the case of brain by Warburg, Posener and Negelein [1924], Loebel [1925] and by Holmes and Holmes [1925 and other papers], and in the case of kidney by Irving [1927, 1928]. In view of such observations the interesting possibility arises that these tissues may contain an activator similar in nature to the substance which can be obtained from yeast. The work reported in this paper comprises a number of observations which arose out of experiments originally undertaken with the purpose of testing this view.

Preliminary results appeared to indicate that brain did contain a factor which could be extracted and which was capable of activating the system (muscle + glucose) with production of lactic acid; and the same seemed to be true, in lesser degree, for kidney. After a series of failures to obtain active extracts, however, it became apparent that the power of activation was dependent upon the presence of intact tissue in the so-called extracts; and when cell-free preparations were used, or when the tissues were finely ground during the process of extraction, in no case was any activating power demonstrable. This finding is in concordance with the state of affairs in muscle tissue itself, in that whole muscle is able to a certain extent to utilise glucose for the formation of lactic acid—a property which is in large degree lost on mincing, and almost completely lost in an aqueous extract.

It would appear then that at least in the instances of muscle, brain and kidney the first stage in the fermentation of glucose is a process involving intact cells, an observation which has been made by Irving for kidney tissue

and for red blood-corpuscles. Although there is no reason to suppose that the series of reactions leading to the production of lactic acid from carbohydrate is the same for muscle and other tissues, the fact that these other tissues are able to activate glucose in such a way that it can be attacked and fermented by the muscle enzymes supports the possibility that this first stage at least may be identical in all three cases.

Methods.

The suspensions of the various tissues employed were made, except where otherwise stated, by chopping the material finely with scissors and razor, after which the chopped mass was thoroughly mixed with water, or more frequently with Ringer's solution, in such a way that 5 g. tissue were equivalent to 30 cc. of suspension. After allowing the coarser particles to settle the suspensions were used without further treatment. At first these operations were carried out at 0°, but this was later found to be quite unnecessary and indeed to have a somewhat detrimental effect on the subsequent activity of the preparations. Fresh tissue was always employed, the brain and kidney being taken from the same rabbit as was used for the making of the muscle extracts. In the case of the kidney the cortex only was used.

Muscle extracts were made exactly as described in a former paper [Case and McCullagh, 1928].

Experimental tubes were set up as follows:

25 cc. muscle extract,
10 cc. 2 % glucose,
10 cc. phosphate buffer (as in former work),
5 cc. activating fluid (or water for controls).

The muscle extract was always added last of all; 15 cc. samples were then mixed with 45 cc. of trichloroacetic acid, and the remainder incubated for 2 hours in a water-bath at 27°. At the end of this period further samples were taken in the same way. *After filtering off the precipitated proteins, 30 cc. of each filtrate were treated with 10 cc. of 10 % lime suspension and 5 cc. of 10 % $CuSO_4$ to remove interfering carbohydrates. Lactic acid was then* estimated on 5 or 10 cc. *of the copper-lime filtrate according to the method* of Friedemann, Cotonio and Shaffer [1927].

Where phosphates were measured, the Briggs modification of the Bell-Doisy method was used.

Table I.

	mg. lactic acid in 15 cc. sample			
	Before incubation		After incubation	
	Schenk	Trichloroacetic	Schenk	Trichloroacetic
1.	5·7	5·9	18·0	18·8
2.	8·1	7·9	27·5	28·3
3.	11·0	12·2	23·5	23·6
4.	6·4	6·6	24·0	25·1

The method of deproteinisation by means of trichloroacetic acid indicated above has been checked against the more laborious Schenk procedure and has been found to give entirely satisfactory results. This is shown by the typical figures given in Table I. In each case the samples were taken from incubation mixtures of muscle extract, buffer and starch.

These are enough to show that the differences which exist between the values obtained by the two methods lie for the most part within experimental error.

Comparison of brain, kidney, etc. with yeast activator.

Table II demonstrates the production of lactic acid from glucose in presence of brain and of kidney. In tubes 5 and 6 the muscle extract had previously been heated at 80° for 5 minutes, so that these tubes are controls showing that the quantities of brain and kidney employed did not alone give rise to the formation of appreciable amounts of lactic acid.

Table II.

	mg. lactic acid in 15 cc. sample		
	Before incubation	After incubation	Change
1. Water control	8·5	9·2	0·7
2. Yeast activator	8·9	23·0	14·1
3. Brain	9·2	17·0	7·8
4. Kidney	8·6	15·2	6·6
5. Brain (+heated muscle) ...	8·0	8·0	0·0
6. Kidney (+heated muscle)	8·2	8·3	0·1

Various other tissues were next tried under the same conditions as brain and kidney. The results are given in Table III.

Table III.

	mg. lactic acid in 15 cc. sample		
	Before incubation	After incubation	Change
1. Water control	9·6	10·2	0·6
2. Brain	9·6	25·3	15·7
3. Kidney	9·1	18·9	9·8
4. Liver	9·5	11·3	1·8
5. Spleen	9·0	9·7	0·7
6. Testis	9·7	10·0	0·3
7. Lung	9·0	10·2	1·2
8. Muscle	10·0	14·2	4·2
9. Blood	9·9	12·8	2·9
10. Brain (+heated muscle) ...	10·1	10·9	0·8
11. Kidney (+heated muscle)	10·4	11·3	0·9
12. Blood (+heated muscle) ...	10·2	10·1	−0·1

Other than brain and kidney, the only tissues which here bring about activation are muscle, as would be expected, and blood. This latter is of interest in view of Irving's [1926] investigations on the glycolysis of red blood-corpuscles. The experiment was repeated, using a suspension of blood-corpuscles which had been centrifuged and washed with saline. The blood was obtained

from a rabbit killed by a blow on the neck, as it was feared that amytal might exert a disturbing influence.

Table IV.

	mg. lactic acid in 15 cc. sample		
	Before incubation	After incubation	Change
1. Water control	8·4	8·6	0·2
2. Blood-corpuscles	8·7	10·9	2·2
3. Blood-corpuscles (+heated muscle)...	8·9	9·2	0·3

The activation by the blood-corpuscles, though not very marked in extent, is nevertheless quite definite.

Table V gives typical results of experiments which were done to illustrate the deleterious effect of grinding and filtering, etc.

Table V.

	mg. lactic acid in 15 cc. sample		
	Before incubation	After incubation	Change
1. Water control	11·3	11·8	0·5
2. Brain chopped (+Ringer)	11·7	28·8	17·1
3. ,, chopped (+water)	11·4	19·6	8·2
4. ,, Ringer suspension centrifuged ...	11·7	14·1	2·4
5. ,, Ringer filtered through muslin ...	11·9	17·7	5·8
6. ,, ground lightly with sand	12·0	14·6	2·6
7. ,, well ground with sand	11·8	12·0	0·2
8. Kidney chopped as usual	12·4	21·9	9·5
9. ,, ,, and centrifuged	11·8	12·5	0·7
10. ,, ground with sand	12·0	11·7	–0·3

It is evident from this that the activation which is brought about by brain and kidney is not dependent upon a soluble or extractable substance, as is the case with yeast; for any process which destroys or removes intact cells has a corresponding effect in diminishing the activating power of the tissue.

Attempts which have been made to prepare alcoholic precipitations and to bring about separation by means of various other precipitants have all proved fruitless.

The effect of cyanide on the activation.

The glycolysis of brain tissue is known not to be affected by the presence of cyanide; in fact this substance is usually added to the buffer solutions used in investigations concerning brain carbohydrate metabolism, in order to prevent oxidation of lactic acid which otherwise takes place to a large extent. Nor does cyanide exert any influence on the production of lactic acid from carbohydrate by muscle extract.

On the other hand, it has been shown by Irving [1927] that the utilisation of glucose by kidney tissue can be completely inhibited by potassium cyanide in small concentration. It seemed of interest therefore to ascertain whether

cyanide would inhibit the activation of glucose + muscle by kidney tissue and not by brain.

First of all the following experiment was performed in order to confirm the fact that while the glycolysis of brain itself is not inhibited by cyanide, that of kidney is.

Tubes were set up thus:

1. 5 g. chopped brain + 10 cc. 2 % glucose + 20 cc. phosphate buffer
2. 5 g. chopped brain ,, ,, ,, + M/500 KCN
3. 5 g. chopped kidney ,, ,, ,,
4. 5 g. chopped kidney ,, ,, ,, + M/500 KCN

Table VI.

	mg. lactic acid in 15 cc. sample		
	Before incubation	After incubation	Change
1.	2·8	1·9	− 0·9
2.	2·9	5·6	+ 2·7
3.	2·0	5·5	+ 3·5
4.	2·3	2·6	+ 0·3

Table VII presents the results of a typical experiment performed with the object of investigating the influence of cyanide on the activations of yeast, brain and kidney.

The incubation tubes were as follows:

1. 25 cc. muscle + 10 cc. glucose + 10 cc. buffer + 5 cc. H_2O
2. ,, ,, ,, 5 cc. yeast activator
3. ,, ,, ,, ,, ,, + M/500 KCN
4. ,, ,, ,, 5 cc. brain
5. ,, ,, ,, ,, + M/500 KCN
6. (heated) ,, ,, ,,
7. (heated) ,, ,, ,, + M/500 KCN
8. 25 cc. muscle ,, ,, 5 cc. kidney
9. ,, ,, ,, ,, + M/500 KCN
10. (heated) ,, ,, ,,
11. (heated) ,, ,, ,, + M/500 KCN

Table VII.

	mg. lactic acid in 15 cc. sample		
	Before incubation	After incubation	Change
1.	5·5	6·4	0·9
2.	5·5	18·2	12·7
3.	5·6	19·0	13·4
4.	5·4	13·3	7·9
5.	5·5	14·0	8·5
6.	5·0	6·2	1·2
7.	5·1	6·5	1·4
8.	5·7	15·7	10·0
9.	5·6	15·4	9·8
10.	5·2	5·4	0·2
11.	5·3	5·4	0·1

From the figures in the foregoing two tables the following facts of importance emerge.

(*a*) Brain glycolysis is not inhibited by M/500 KCN.

(*b*) Kidney glycolysis on the other hand is prevented by this concentration of cyanide.

(*c*) The activation of the system muscle + glucose by either yeast, brain or kidney is unaffected by cyanide.

The conclusion must be drawn from this that the stage at which cyanide inhibits glycolysis by kidney tissue alone is one subsequent to the preliminary process of activation. This is what might be expected, in that there is no reason to suppose that the mechanism of activation involves any oxidative process.

The influence of fluoride.

It is well known that when fluoride is present in an incubated mixture of starch or glycogen and muscle extract, the breakdown of hexosephosphates but not their formation is prevented; consequently no lactic acid appears, and free phosphate rapidly disappears.

In the course of some experiments with yeast activator it was found that the addition of fluoride to the system (muscle + glucose + activator) suppressed not only the formation of lactic acid, but in addition the synthesis of hexosephosphate, so that no change in either carbohydrate, free phosphate or lactic acid was observable at the end of the incubation.

The effect of fluoride on the changes occurring in presence of brain and kidney was therefore tried, with the results shown in Table VIII.

The contents of the tubes were as follows:

1. Muscle + starch + buffer + 5 cc. H_2O
2. ,, ,, ,, ,, + $M/50$ NaF
3. ,, glucose ,, 5 cc. yeast activator
4. ,, ,, ,, ,, ,, + $M/50$ NaF
5. ,, ,, ,, 5 cc. brain
6. ,, ,, ,, ,, + $M/50$ NaF
7. ,, ,, ,, 5 cc. kidney
8. ,, ,, ,, ,, + $M/50$ NaF

(The usual controls with heated muscle extract were performed, but are omitted for the sake of clarity.)

In addition to lactic acid determinations, free phosphate was estimated before and after incubation in 3 cc. of the trichloroacetic acid filtrate.

Table VIII.

	mg. lactic acid in 15 cc. sample			mg. P as free phosphate in 3 cc. sample		
	Before	After	Change	Before	After	Change
1.	7·8	28·2	20·4	0·82	0·77	−0·05
2.	7·8	8·1	0·3	0·83	0·12	−0·71
3.	7·9	25·9	18·0	0·94	0·62	−0·32
4.	7·8	8·2	0·4	0·94	0·92	−0·02
5.	8·0	17·5	9·5	0·85	0·80	−0·05
6.	7·9	7·6	−0·3	0·86	0·39	−0·47
7.	8·1	16·0	7·9	0·84	0·72	−0·12
8.	8·0	8·2	0·2	0·85	0·25	−0·60

This demonstrates that the action of fluoride, when muscle extract is utilising glucose in presence of brain or kidney, is similar to its effect in the case where muscle extract alone is breaking down starch or glycogen; *i.e.* the hydrolysis of phosphoric esters is prevented: whereas when yeast acti-

vator is employed, fluoride prevents even the synthesis of hexosephosphate from glucose.

The effect of phloridzin etc. upon yeast activation.

Dann and Quastel [1928] showed that the zymin fermentation of glucose was greatly retarded by phloridzin and the corresponding phenol, phloroglucinol; glucosides other than phloridzin did not exhibit this phenomenon.

It seemed conceivable that phloridzin and phloroglucinol might also exert this inhibitory effect upon the activation of glucose by yeast prior to glycolysis. If this were found to be the case it would be evidence that the first change undergone by glucose is the same in the cases of alcoholic fermentation and glycolysis. The following experiment however shows the entire absence of any effect of these substances. In addition to the usual contents, each incubation vessel contained 10^{-3} gram-molecule of the material under test.

Table IX.

	mg. lactic acid in 15 cc. sample		
	Before incubation	After incubation	Change
1. Water control	5·3	5·7	0·4
2. Yeast activator	5·5	26·2	20·7
3. ,, + phloridzin	5·2	26·9	21·7
4. ,, + phloroglucinol	5·7	27·4	21·7
5. ,, + amygdalin	5·1	25·5	20·4
6. ,, + salicin	5·5	26·0	20·5

From these results one must conclude either that the first or activating stage differs in the two processes or that the inhibition observed by Dann and Quastel occurs at some later period in the fermentation.

The effect of tumour tissue on muscle glycolysis.

It has been stated by Waterman [1924, 1925] and by Kraut and Bumm [1928] that cell-free extracts of tumours are capable of accelerating the glycolysis of certain normal tissues; these claims have however been criticised on various grounds by Brooks and Jowett [1928]. It was thought worth while to investigate the influence, if any, of such extracts upon the fermentation of glucose by muscle extracts.

Experiments which were carried out with the Rous chicken sarcoma indicated that, in common with the other tissues that have been tried, cell-free extracts of this tumour are not able to activate the utilisation of glucose by muscle extract; whereas suspensions of the comparatively uninjured sarcoma tissue do possess this power.

The following are typical of a number of experiments which were made:

1. Muscle + glucose + buffer + H_2O
2. ,, ,, ,, + chopped tumour suspension
3. ,, ,, ,, + ,, ,, centrifuged
4. ,, ,, ,, + tumour ground with Ringer's solution in mortar
5. ,, ,, ,, + ,, ,, ,, and centrifuged
6. ,, ,, ,, + tumour ground with sand
7. (heated) ,, ,, + chopped tumour suspension

The tumours were always obtained from recently-killed fowl. Lactic acid estimations before and after incubation are given in Table X.

Table X.

	mg. lactic acid in 15 cc. sample		
	Before incubation	After incubation	Change
1.	6·8	7·9	1·1
2.	7·2	28·0	20·8
3.	7·2	9·7	2·5
4.	7·2	13·5	6·3
5.	7·0	9·2	2·2
6.	7·0	8·3	1·3
7.	6·7	7·4	0·7

In this connection it is pertinent to note that, in common with other tissues, hashing of cancer tissue diminishes its glycolytic activity, while freezing, grinding or extraction destroys this property entirely [Barr, Ronzoni and Glaser, 1928].

Discussion and summary.

It is shown that in the presence of small amounts of brain and kidney tissue, a muscle enzyme preparation which alone is incapable of any appreciable degree of glycolysis is enabled to ferment glucose with production of lactic acid. This activation is compared with that brought about by addition to the system of the substance obtained by Meyerhof from yeast. Evidence is further adduced to show that, contrary to what occurs in the case of the yeast preparation, the activation of glucose by brain and kidney is associated with the presence of intact cell-structures. It is not suggested that this property is one specific for the tissues mentioned; these were chosen because they are among the tissues with the highest glycolytic activity, and it is probable that their activating power differs from that of other tissues only in degree.

The apparent correlation that exists between glycolysis and the presence of uninjured cells is in harmony with the views generally held concerning glucose metabolism. It is by no means certain that from any tissue a cell-free extract can be obtained which has the power of fermenting glucose, though at different times claims of this nature have been made; Stiven [1928], for example, has recently described the preparation of a cell-free extract of muscle which is stated to produce lactic acid from glucose. Even so, however, the fact remains that in the cases of all tissues that have been examined, partial or complete destruction of the cell-structure is concomitant with corresponding diminution in glycolytic power, from which the inference may be drawn that these structures are essential for at least a major part of the glycolytic activity possessed by the tissues.

A brief study of one particular type of tumour tissue, viz. the Rous sarcoma, has indicated that here also the intact tissue is necessary for the

activation of glucose, and it has not been found possible to extract any substance which possesses this property.

As to the nature and significance of the activation brought about by these tissues or by yeast activator, there is little that can be said. There is at present no explanation for the fact that a muscle extract which vigorously breaks down glycogen to lactic acid should be practically inactive when the substrate is glucose, other than the postulate that the hexose molecules which form the structure of the polysaccharide are present in some hypothetical "active" condition in virtue of which they are accessible to the muscle enzymes. A discussion of this aspect of the question cannot here be entered upon, but the subject is clearly relevant to a study of glucose activation.

I wish to express my indebtedness to Sir F. G. Hopkins and Mr J. B. S. Haldane for their interest and advice, and to the Department of Scientific and Industrial Research for a grant.

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XXX. PHOSPHORIC ESTERS IN ALCOHOLIC FERMENTATION.

I. THE SEQUENCE OF THE FORMATION OF PHOSPHORIC ESTERS AND CARBON DIOXIDE IN FERMENTATION BY DRIED YEAST.

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AFTER Harden and Young [1906] had shown the importance of phosphates in fermentation, discovered hexosediphosphate and advanced the equations

(1) $2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$,

(2) $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$,

Harden and Robison [1914] isolated hexosemonophosphoric ester from fermenting yeast juice. Realising that this would complicate the original equation, Harden and Henley [1927] investigated the relationship between the carbon dioxide and the hexose mono- and di-phosphates formed when inorganic phosphate is added to fermenting zymin and yeast juice. They found the ratio of the two esters to be very variable, but the amount of carbon dioxide evolved was always somewhat less than the equivalent of the total phosphorus esterified. On the average of the results obtained with zymin (acetone yeast), the carbon dioxide was slightly more than equivalent to the hexosediphosphoric ester formed. These results can be taken to support the view of Meyerhof and Lohman [1927] who suggested that hexosemonophosphate is first formed without liberation of any carbon dioxide, but later reacts to liberate carbon dioxide and produce hexosediphosphate. With some yeast juices the amount of carbon dioxide is much greater than the amount of diphosphoric ester, though it is never more than equivalent to the total phosphorus esterified.

Recently, Robison and Morgan [1928] have isolated a disaccharidemonophosphate, trehalosemonophosphoric ester, from the products of fermentation of sugar by dried yeast. Up to the present trehalosemonophosphoric ester has only been isolated from dried yeast though there have been indications of its occurrence in the products of fermentation by zymin.

In the present paper the sequence of ester production and carbon dioxide evolution and the ratios of the gas to the esters formed by dried yeast are examined.

EXPERIMENTAL METHOD.

The method used in the first experiments was almost identical with that described by Harden and Henley [1927]. The precipitation of the barium salt of the diphosphoric ester was modified by adding 10 % alcohol in order

to obtain more complete precipitation. Later investigation indicated, however, that this addition of alcohol caused a slight precipitation of the monophosphate, so that it did not give a perfectly clean separation. The free and combined phosphate in the solutions were estimated by the colorimetric method developed by Martland and Robison [1926].

The carbon dioxide was measured by the method of Harden, Thompson and Young [1910], and was corrected for the basic rate of fermentation and for the gas evolved owing to the production of the relatively acid esters from the free phosphate. For this purpose in all cases the gas evolved on addition of the trichloroacetic acid was measured. The difference in the amounts of gas evolved by this acid at various stages of the fermentation indicates the extent of this acid production and the correction is applied to the volume of gas liberated by the reaction of the phosphate.

The influence of the concentration of phosphoric esters on the basic rate of fermentation.

The volume was also corrected for the amount of gas which would have been formed by the normal fermentation without addition of phosphate. This last is the basic rate of fermentation caused by the steady hydrolysis of the hexosediphosphate (see equation 2). It was possible to show that in the case of many dried yeasts this basic rate of fermentation varied considerably with the concentration of phosphoric esters present. It was therefore necessary to investigate the relationship between the basic rate of fermentation and the concentration of phosphoric esters.

Several lots of 4 g. dried yeast + 25 cc. of 10 % fructose solution + 0·2 cc. of toluene were incubated at 30° and the gas evolved was measured. When equilibrium was attained (after at least an hour) various amounts of 0·6 *M* dipotassium hydrogen phosphate solution were added to the different preparations. After all the free phosphate had reacted and equilibrium was again attained the basic rate of fermentation was carefully measured and the fermentation then stopped in the usual way with trichloroacetic acid, and the phosphoric esters were estimated in the trichloroacetic acid filtrate. The results of such an experiment are to be found in Table I and in Fig. 1.

Table I. *The relation between phosphoric ester concentration and the basic rate of fermentation.* K_2HPO_4 *added.*

Exp. 44. Dried yeast of 9. iii. 28.

Molecular concentrations			Basic or hydrolysis rate	Ratios		
Total P	P as diphosphate	P as monophosphate	cc. per 5 min.	P as diphosphate / P as monophosphate	Basic rate / P as monophosphate	Basic rate / P as diphosphate
0·072	0·061	0·011	2·5	5·5	227	41
0·098	0·084	0·014	3·0	6·0	215	36
0·107	0·091	0·016	4·3	5·7	268	47
0·152	0·136	0·017	4·9	8·0	290	36
0·188	0·171	0·017	5·0	10·0	294	29
0·228	0·211	0·017	5·0	12·4	296	24

Except that no large amount of carbon dioxide was evolved and that equilibrium was quickly attained, the addition of hexosediphosphate instead of potassium phosphate gave almost identical results as regards the equi-

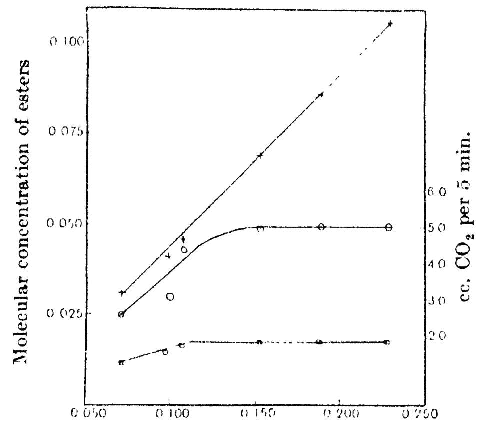

Fig. 1.

—×—×—×— Diphosphoric ester.

—o—o—o— Monophosphoric esters.

—o—o—o— Hydrolysis or basic rate (cc. CO_2 per 5 min.).

librium attained and the change in basic rate of fermentation. Such an experiment is shown in Table II and in Fig. 2.

Table II. *The relation between the phosphoric ester concentration and the basic rate of fermentation after the addition of hexosediphosphate.*

Exp. 45. Dried yeast of 20. ii. 28.

Molecular concentrations			Hydrolysis rate	Ratios		
Total P	P as di-phosphate	P as mono-phosphate	cc. per 5 min.	P as diphosphate / P as monophosphate	Basic rate / P as monophosphate	Basic rate / P as diphosphate
0·071	0·061	0·010	3·9	6·1	390	64
0·080	0·068	0·012	4·3	5·7	358	63
0·087	0·074	0·013	4·7	5·7	362	64
0·102	0·088	0·014	5·3	6·3	382	60
0·119	0·105	0·014	6·1	7·5	434	58
0·131	0·116	0·015	5·8	7·7	387	50
0·163	0·149	0·014	6·0	10·6	428	40

These results are in agreement with the earlier work of Harden and Young [1910], who found that the addition of phosphate to a fermenting mixture of yeast juice and sugar containing very little phosphate, caused a greatly increased amount of fermentation. The increase was much greater than the carbon dioxide equivalent of the added phosphate, and it was probably due in part to the increased basic rate caused by the added phosphate. It is therefore probable that there is a necessary phosphoric ester concentration for the maximum basic rate of fermentation of yeast juice as well as of dried

yeast. Yeast juice seems to require less of this phosphate than does dried yeast.

In calculating the amount of carbon dioxide evolved by the reaction of inorganic phosphate, the basal fermentation is therefore assumed to proceed at a rate varying with the amount of esterification that has occurred. Thus up to a certain concentration (varying slightly with the preparation) the basic rate of fermentation is assumed to be proportional to the concentration of esterified phosphate. At the beginning of the reaction the rate is assumed to be the same as that observed before the addition of phosphate. After the time of complete esterification it is taken as being the same as that of the final basic rate. At a point when half the phosphate is esterified it is assumed

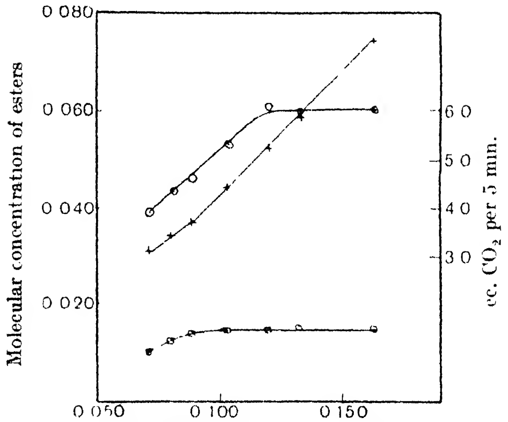

Fig. 2.

Diphosphoric ester.
Monophosphoric esters.
Hydrolysis or basic rate (cc. CO_2 per 5 min).

to be half-way between these two rates, and so on. If the basic rate is assumed to be that which occurred before the addition of phosphate, then the calculated amount of carbon dioxide liberated by the phosphate will be too high. If it is taken as the final basic rate then the correction will be too large and the final figure for the carbon dioxide will be too low. The different methods of calculating the basic rate may cause differences of as much as 10 % in the calculation of the amount of gas evolved. It is deduced from the previous experiments that the assumption of the basic rate being proportional to the esterified phosphorus gives the most accurate value for the carbon dioxide.

The equilibrium of the phosphoric esters.

These results also show that up to a certain concentration of phosphate, there is proportionality between the concentrations of hexosediphosphate, monophosphate and the hydrolysis rate. Above this critical concentration, the

amount of monophosphate and the hydrolysis rate remain constant. The significance of the fact that the basic rate and the monophosphoric ester concentration have the same critical concentration is not quite clear, although it possibly indicates that the slowest reaction in the system is the formation of the monophosphoric ester. The rate of the formation of this ester increases up to a certain point with increase in the concentration of hexosediphosphate, but beyond the critical point the enzyme acting on the diphosphate becomes saturated with this substrate. The limiting factor after this point is not the amount of ester present, but rather the amount of enzyme.

The monophosphoric ester isolated from dried yeast fermentations in equilibrium, on analysis and determination of the optical rotation and reducing power, seems to contain 70 % of its phosphorus bound as trehalosemonophosphate.

Hexosediphosphate added to dried yeast fermenting fructose is in part converted into trehalosemonophosphate, thus maintaining the equilibrium ratio between the two esters. If this equilibrium is true, it should also be obtained when trehalosemonophosphate is added to the fermenting mixture. In order to test this, a solution of the potassium salt of trehalosemonophosphate was added to fermentations which were stopped in the usual way during the reaction. The changes in the esters are shown in Table III. The change appeared to be accompanied by a slight increase in carbon dioxide evolution.

Table III. *Phosphoric ester equilibrium after the addition of trehalosemonophosphate to dried yeast and fructose.*

Exp. 81. Dried yeast of 9. iii. 28.

Time after addition	Molecular concentrations expressed as P: Diphosphate	Monophosphate	P as diphosphate / P as monophosphate
No addition	0·078	0·013	6·0
0	0·078	0·020	3·9
5	0·080	0·018	4·5
10	0·082	0·016	5·1
15	0·083	0·015	5·6
30	0·084	0·014	6·0

Thus trehalosemonophosphate appears to be rapidly hydrolysed by fermenting dried yeast with excess sugar and the usual equilibrium ratio between the two esters is soon reached.

The equilibrium is reached in about the same time if hexosediphosphate is added to the fermenting mixture, in place of trehalosemonophosphate, as is shown in Table IV. In this experiment a solution of the potassium salt of hexosediphosphate was added to the fermenting preparations which were stopped at various intervals after the additions. The carbon dioxide evolution was measured, but the large change in the basic rate and the period required for the completion of the reaction made it impossible to calculate the extra carbon dioxide evolved, if indeed any was evolved.

Table IV. *The rate of change in phosphoric esters after the addition of hexosediphosphate to dried yeast and fructose.*

Exp. 83. Dried yeast of 9. iii. 28.

Time after addition	Molecular concentration expressed as P: Diphosphate	Molecular concentration expressed as P: Monophosphate	P as diphosphate / P as monophosphate
No addition	0·101	0·016	6·3
0	0·140	0·016	8·8
10	0·136	0·018	7·5
20	0·131	0·020	6·6
30	0·130	0·020	6·5
40	0·133	0·020	6·6

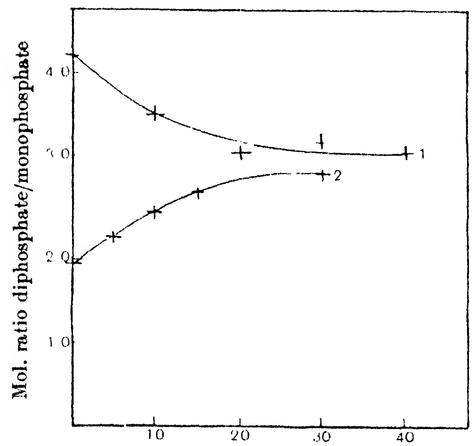

Fig. 3. In curve 1 hexosediphosphate was added; in curve 2 trehalosemonophosphate was added to dried yeast fermenting fructose.

These last two experiments are shown graphically in Fig. 3, where it can be seen that the equilibrium of the two esters is a true equilibrium, which is attained from either side at almost the same rate. The occurrence of this equilibrium ratio of the esters probably indicates that one of these esters is derived from the other.

The lag between esterification and carbon dioxide evolution.

In order to study the relations of the formation of the various products of the reaction of phosphate with dried yeast, the same amount of phosphate was added to several preparations which were stopped at various times during the reaction. The carbon dioxide evolved was measured and the free phosphate and phosphoric esters in solution were estimated. The carbon dioxide was corrected for the amount evolved by the formation of the relatively acid phosphoric esters and for the basic hydrolysis rate.

The fully corrected results of typical experiments of this kind are shown

in Tables V, VI and VII and in Fig. 4. In the tables the ratios of the P in the form of the two esters and of the amount of carbon dioxide formed to the amount of P as diphosphoric ester, and to the amount of P esterified plus the amount of P as monophosphate (P as diphosphate plus twice the P as monophosphate) are given.

Table V. *The time relations of the products of the reaction of phosphate with dried yeast.*

Exp. 42. 2·5 cc. 0·6 *M* K_2HPO_4 added to 4 g. dried yeast + 25 cc. 10 % fructose solution. Basic rate before addition 2·5 cc.; after, 3·0 cc. per 5 min. The mono- and di-phosphate are expressed in terms of P.

Time min.	Mol. conc. Mono-phosphate	Mol. conc. Di-phosphate	Mol. conc. of substances formed Di-phosphate	Mol. conc. of substances formed CO_2	Ratios $\frac{\text{Diphosphate}}{\text{Monophosphate}}$	Ratios $\frac{CO_2}{\text{P esterified}}$	$\frac{CO_2}{\text{P esterified + P as monophosphate}}$
0	0·026	0·078	– –	—	3·0	—	—
10	0·028	0·101	0·023	0·024	3·6	0·96	0·89
20	0·032	0·105	0·027	0·046	3·3	1·40	1·18
30	0·033	0·107	0·029	0·046	3·2	1·28	1·07
40	0·031	0·109	0·031	0·046	3 5	1·28	1 12
50	0·033	0·107	0·029	0·046	3 2	1·28	1·07

Table VI.

Exp. 32 (cf. Fig. 3). 5 cc. 0·6 *M* K_2HPO_4 added to 4 g. dried yeast + 25 cc. 10 % fructose solution. Basic rate before addition 4·4 cc.; after, 6·5 cc. per 5 min. The mono- and di-phosphate are expressed in terms of P.

Time min.	Mol. conc. Mono-phosphate	Mol. conc. Di-phosphate	Mol. conc. of substances formed Di-phosphate	Mol. conc. of substances formed CO_2	Ratios $\frac{\text{Diphosphate}}{\text{Monophosphate}}$	Ratios $\frac{CO_2}{\text{P esterified}}$	$\frac{CO_2}{\text{P esterified + P as monophosphate}}$
0	0·011	0·073	– –	–	6·6	—	—
15	0·009	0·147	0·076	0·061	15 2	0·85	0·88
30	0·010	0·170	0·097	0·098	17·0	1·01	1·02
45	0·023	0·156	0·083	0·113	6·8	1·18	1·05
60	0·025	0·154	0·081	0·113	6·2	1·18	1·03
75	0·021	0·158	0·085	0·113	7·5	1·18	1·07
90	0·025	0·154	0·081	0·113	6·2	1·18	1·03

Table VII.

Exp. 62. 10 cc. 0·6 *M* K_2HPO_4 added to 4 g. dried yeast + 25 cc. 10 % fructose solution. Basic rate before addition 4·4 cc.; after, 6·6 cc. per 5 min. The mono- and di-phosphate are expressed in terms of P.

Time min.	Mol. conc. Mono-phosphate	Mol. conc. Di-phosphate	Mol. conc. of substances formed Di-phosphate	Mol. conc. of substances formed CO_2	Ratios $\frac{\text{Diphosphate}}{\text{Monophosphate}}$	Ratios $\frac{CO_2}{\text{P esterified}}$	$\frac{CO_2}{\text{P esterified + P as monophosphate}}$
0	0·016	0·064	—	—	4·0	—	—
5	0·016	0·078	0·014	0·009	4·9	0·65	0·65
10	0·019	0·097	0·033	0·018	5·1	0·50	0·46
15	0·023	0·111	0·047	0·036	5·1	0·68	0·61
40	0·034	0·196	0·132	0·149	5·7	1·00	0·89
50	0·034	0·204	0·140	0·167	6·0	1·06	0·96
75	0·034	0·204	0·138	0·168	6·0	1·05	0·97

This type of experiment was carried out many times, with different samples of dried yeast and with different amounts of added phosphate, and

similar results were always obtained. Examination of these observations shows that the hexosediphosphoric ester appears as the first product of the reaction and that there is a considerable lag between esterification and the evolution of carbon dioxide. This is not due to physical causes such as supersaturation, because such would be accounted for in the correction applied for the production of acid esters from inorganic phosphate. In the presence of free phosphate, therefore, the esterification proceeds at a higher rate than the reaction, whatever it may be, which results in the formation of alcohol and carbon dioxide. At the end of the reaction the carbon dioxide production overtakes the ester formation and finally somewhat more gas is evolved than is equivalent to the reacting phosphate.

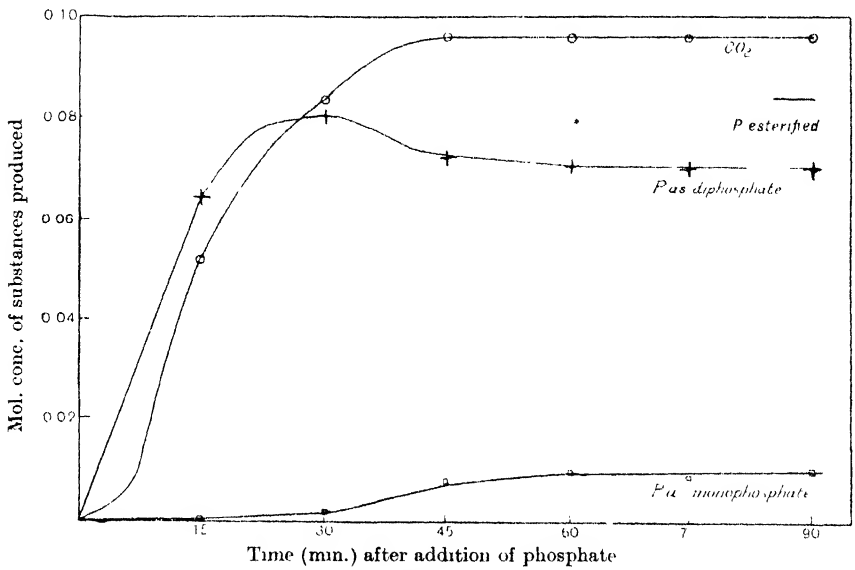

Fig. 4.

A similar delay in carbon dioxide evolution after the addition of phosphate has been indicated by Euler and Johansson [1913]. They considered that the activation of the sugar, the formation of hexosediphosphate and then of a triosemonophosphate were stages in the reaction of phosphate which all occurred before the evolution of carbon dioxide in the reaction of phosphate with dried yeast in the presence of dextrose.

The ratio of carbon dioxide to phosphoric esters.

In all cases in which the gas evolved from the complete reaction of phosphate was measured, it was found to be somewhat more than equivalent to the phosphate. On the other hand maceration extract, yeast juice and zymin, so far as at present known, tend to give less than the equivalent amount of carbon dioxide from added phosphate [Harden and Henley, 1927; Kluyver

and Struik, 1928]. Coupled with this is the fact that dried yeast alone gives a monophosphate fraction consisting mainly of trehalosemonophosphate. In all the experiments, the ratios of carbon dioxide to the total P esterified, and to the total P esterified plus the P as monophosphate were calculated. The results of over 50 such determinations, the averages of which are shown in Table VIII, point to equivalence between the carbon dioxide and the total P esterified plus the P as monophosphate, *i.e.* to the amount of P as diphosphate plus twice the amount of P as monophosphate. Thus the phosphorus finally bound as monophosphate appears to react twice. This may possibly be explained by a change of the diphosphoric ester into the monophosphoric ester and free phosphate at a relatively high rate, the latter then reacting again to liberate carbon dioxide and form hexosediphosphate.

The ratio of carbon dioxide to esterified phosphorus appears to vary with the amount of reacting phosphate, being lower with large amounts of phosphate; this may be due to two causes. Firstly, the addition of large amounts of phosphate causes an inhibition in the carbon dioxide production, probably due to a lowering of the basic rate of fermentation so that the observed corrected amount of carbon dioxide will be too low. Smaller amounts of phosphate produce no such inhibition and there is consequently no error of this kind. Secondly, with small amounts of phosphate there is a relatively larger amount of monophosphate formed, so that, if the ratio of carbon dioxide to phosphorus esterified plus the amount of P as monophosphate remains at unity, then the ratio of carbon dioxide to the phosphorus esterified will necessarily be high.

Table VIII. *The ratio of carbon dioxide evolved to phosphoric esters produced on adding potassium phosphate to dried yeast fermenting fructose.*

Yeast	cc. 0·6 M K_2HPO_4 added to 4 g. dried yeast	No. of experiments	Average ratios: $\frac{\text{Carbon dioxide}}{\text{P esterified}}$	Average ratios: $\frac{\text{Carbon dioxide}}{\text{P esterified + P as monophosphate}}$
20. II. 28	2·5	7	1·16	0·98
	5·0	14	1·14	1·02
	7·5	2	1·07	1·00
	10·0	1	1·06	1·00
9. III. 28	2·5	8	1·22	1·01
	5·0	8	1·04	1·00
	7·5	2	1·05	1·02
	10·0	7	1·05	0·99
11. VI. 28	5·0	3	1·16	1·01
Scotch bottom yeast	5·0	2	1·08	1·00
	Total	54	Average 1·12	1·00

The maximum error in the determination of the ratio of carbon dioxide to esterified phosphate is probably about 5 % in each single experiment. The amount of phosphate can be measured easily to 1 % but the carbon dioxide cannot be determined to nearer than 2 or 3 %. The determination of the monophosphate may have a 10 % error, but, as it forms only one-seventh

of the total phosphate, the error will only influence the final ratio by 2 %. The average of 50 determinations of the ratio of carbon dioxide to the P esterified plus the amount of P as monophosphate is 1·00, and this is probably accurate to within 2 %.

Discussion.

The ratio of the various enzymes in dried yeast is such that under equilibrium conditions (*i.e.* when a steady rate of carbon dioxide evolution is attained) the esterified phosphate consists mainly of hexosediphosphate together with a little monophosphoric ester, which is largely the ester of the disaccharide trehalose. The equilibrium attained after the addition of phosphate (below a certain critical concentration) gives the same ratio of the phosphoric esters. There appears to be true equilibrium between the esters, depending upon the amounts of the various enzymes present in the preparation. If relatively large amounts of phosphate are added (*i.e.* above the critical concentration) the ratio of hexosediphosphate to monophosphate is increased. This can be explained by the saturation of the enzyme acting on the diphosphate, so that increases in its concentration beyond a limiting value do not affect the rate of its decomposition.

In presence of dried yeast the monophosphoric esters are produced late in the reaction with phosphate—after much hexosediphosphoric ester has been formed—and the phosphorus bound in this form appears to liberate two equivalents of carbon dioxide.

The significance of trehalosemonophosphoric ester in the process of fermentation is not yet clear but it is hoped that it will be further elucidated in a later communication.

Summary.

Experiments on the action of phosphates and observations on the esters occurring in fermenting dried yeast preparations have given the following results.

(1) Up to a limiting concentration of phosphoric esters, the basic rate of fermentation varies with the concentration of phosphorus present as phosphoric esters. Up to this same concentration the ratio of diphosphoric ester to monophosphoric ester remains constant, but above the limiting concentration the relative amount of diphosphoric ester increases.

(2) The addition of inorganic phosphate to dried yeast fermenting fructose is followed by esterification and liberation of carbon dioxide, but there is a considerable time lag between esterification and the evolution of carbon dioxide.

(3) The carbon dioxide liberated by phosphate is somewhat more than equivalent to the added phosphate, but is approximately equivalent to the phosphorus esterified plus the amount of P as monophosphate formed (*i.e.* the P as diphosphate plus twice the P as monophosphate).

(4) In fermentations by dried yeast in presence of phosphate the monophosphoric esters are formed late in the reaction. Under the conditions described in this paper these esters consist mainly of trehalosemonophosphate.

Throughout this work my indebtedness to Prof. A. Harden and to Dr R. Robison will be obvious, and I wish to thank them for the constant help they have given me during the progress of the research.

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XXXI. THE EQUATION OF ALCOHOLIC FERMENTATION. II.

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EVIDENCE has been accumulating for some time that with certain yeast preparations a very large proportion of the phosphorus esterified during the fermentation of a mixture of sugar and phosphate is found at the close of the fermentation in the form of a monophosphoric ester. In his early experiments with yeast juice Robison [1922] obtained as much as 15–30 % of the total esterified P in the form of monophosphate, equivalent to a molecular ratio of diphosphate/monophosphate of 2·8 to 1·6, but in later experiments, Robison and Morgan [1928], as much as 47–50 % (molecular ratio 0·5) has been observed. These results were obtained by repeated additions of phosphate to the fermenting mixture. In our previous paper two low ratios (0·61 and 0·72) were noted in this laboratory. The general experience drawn from a large number of experiments is that with dried top yeast from a brewery high ratios are almost invariably obtained, whereas with yeast juice the results are much more variable and low ratios are frequent.

The matter is further complicated by the fact that the monophosphate formed by dried yeast (brewer's top yeast) consists very largely of trehalosemonophosphate [Robison and Morgan, 1928] whereas this substance has not yet been detected among the products from yeast juice. Kluyver and Struik [1928], working at Delft with maceration extract prepared from dried yeast, have also obtained considerable proportions of monophosphate and consider that the ratio in which di- and mono-phosphate are finally present depends on the dilution of the extract, more monophosphate (up to 80 % of the total esterified) being obtained with the more dilute juice.

As pointed out in the previous paper the production of monophosphate necessitates some modification of the equation originally proposed by Harden and Young in which only the formation of diphosphate is taken into account. In that paper it was shown that, notwithstanding the considerable variation in the proportions of di- and mono-phosphate produced, the ratio of CO_2/P esterified was approximately constant at about 0·9. It was suggested that this indicated that about 10 % of the phosphate was esterified without production of carbon dioxide. As regards the ratio of CO_2/hexosediphosphate it was pointed out that small deviations from the theoretical value of 2 might be accounted for on the ground of partial hydrolysis of the diphosphate with

formation of monophosphate. No experimental evidence in favour of this supposition is available and, as was then remarked, this explanation is inapplicable when a large proportion of monophosphate is formed.

In order to obtain more information on all these points a further series of experiments has been made in which (*a*) dried yeast of various origins, (*b*) maceration extracts made from two of the samples of dried yeast used in (*a*), and (*c*) yeast juice were employed.

Experimental.

The methods adopted were described in the previous paper [1927]. The estimation of the proportions of mono- and di-phosphoric esters produced has received further consideration. It has been found by Robison that the precipitate of barium salts produced by adding baryta and barium acetate to the trichloroacetic acid filtrate from the fermented liquid may not contain the whole of the barium hexosediphosphate present. A certain amount may remain in solution along with the monophosphate. This is precipitated by the addition of one-ninth of a volume of alcohol, so that greater accuracy is obtained by estimating the organic phosphorus in the alcoholic filtrate and calculating this as monophosphate. Even with this modification the estimation is not susceptible of great accuracy, as a small amount of the monophosphate is sometimes precipitated under these conditions.

A second possible source of error lies in the fact that in the presence of free phosphate, hexosediphosphate is almost completely removed from solution by the treatment with baryta and barium acetate, so that no further precipitate is produced by the addition of alcohol. Since the baryta treatment at the commencement of the experiment is carried out in presence of free phosphate, it is essential to precipitate with 10 % alcohol at the end of the experiment, at which period no free phosphate is present, in order to get comparable results. In the work recorded in the previous paper the alcohol method was not employed, so that the amounts of monophosphate there recorded are probably all somewhat too high and those of diphosphate somewhat too low.

The samples taken before and after esterification are treated in the same way and the numbers quoted below represent the differences between the amounts present at the beginning and end of the esterification period.

Correction for normal rate of fermentation. It was pointed out in the previous paper that the steady rate of fermentation at the end of the experiment is usually higher than at the commencement, owing to the increased concentration of esterified phosphate. Boyland [1929] has found that with dried yeast the steady rate increases with the total esterified phosphorus up to a certain point and then remains constant. It follows from this that the correction to be applied should vary during the process of esterification. In the experiments described by Boyland, in which periodic estimations of esterified

Table I.

Exp. No.	Material used	Sugar	P: cc. 0·6 M K_2HPO_4	P: mg. P added	CO_2 cc. at N.T.P.: Correction for combined CO_2 cc.	CO_2 cc. at N.T.P.: Correction for hydrolysis cc.	CO_2 cc. at N.T.P.: Corrected vol. cc.	Duration. No. of 5 min. periods	mg. P as Hexose diphosphate	mg. P as Hexose monophosphate	CO_2/combined P	CO_2/diphosphate	CO_2/monophosphate	Diphosphate/monophosphate
(a) *Dried yeast:*														
80	D.Y. 14. 6. 27	Fructose	10	180	21·5	63·1	135·4	14	159·8	15·1	1·07	2·35	12·4	5·29
82	,,	,,	15	272	35·3	69·5	189·9	15	233·9	25·6	1·01	2·25	10·3	4·56
84 *a*	,,	Glucose	8	144	15·4	68·5	101·3	16	—	—	0·97*	—	—	—
b	,,	Gl. + acetaldehyde	8	144	15·4	47	109·4	10	—	—	1·05†	—	—	—
85 *a*	,,	Glucose	8	144	17·7	65·3	100·9	17	135·8	10·8	0·95	2·06	12·9	6·3
b	,,	Gl. + acetaldehyde	8	144	17·7	52	112·8	14	134·3	12·3	1·06	2·33	12·7	5·45
93	D.Y. 1. 12. 27	Fructose	10	180	27	108·5	129·8	15	166·5	20·5	0·96	2·16	8·76	4·06
94	,,	,,	10	180	23·5	95	126·1	16	161·6	21·4	0·95	2·16	8·15	3·77
98	D.Y., Y. III	,,	10	164·7	23·6	82·4	131·5	15	129·2	36·3	1·09	2·82	5·01	1·78
(b) *Maceration extracts:*														
92	Maceration ext. D.Y. 1. 12. 27	Fructose	10	180	24	69·2	126	16	107·5	72·5	0·97	3·24	2·41	0·74
97	,,	,,	10	164·7	25·5	65·2	115·3	15	22	140	0·98	11·1	1·49	0·08
99	Maceration ext. D.Y., Y. III	F. + acetaldehyde	10	164·7	23·9	84·1	105·3	18	163·2	5·2	0·86	1·79	28·1	15·6
(c) *Yeast juice:*														
100	Yeast juice	Fructose	10	163·3	27·2	42·2	106	12	151	12·6	0·90	1·94	11·6	5·9
101	,,	,,	10	163·3	26·5	45·2	105·9	13	118·4	43·9	0·90	2·48	3·33	1·35
102	,,	,,	10	160·5	22·1	16·1	109·5	11	124	39·5	0·93	2·44	3·84	1·57

* Total esterified P = 144·5 mg.

† Total esterified P = 143·8 mg.

phosphate were made, it has been possible to apply a suitable correction at each point. The result of this is that his total correction is less than that calculated from the final rate only and his residual volume of gas correspondingly greater. The difference may amount to as much as 10 %, but varies very greatly with the conditions of the experiment, and is much less for yeast juice and zymin than for dried yeast. In the table of results, as in the previous one, the final rate only has been used, as we have no intermediate observations on which to base any modification. Our final volumes of CO_2 and ratios of CO_2/P esterified would therefore be expected to be somewhat lower than those obtained by Boyland.

Results.

Experiments have been carried out with dried yeast, two samples of which had been prepared at different times from mild ale yeast and one from a baker's yeast of Dutch origin: two experiments were made with maceration extract prepared from one of the mild ale dried yeasts and one with maceration extract from the Dutch baker's yeast; finally three experiments were made with yeast juice from mild ale yeasts. The results are given in Table I.

Discussion.

Ratio of CO_2 to total P esterified.

(*a*) *Dried yeast.* The average value for this ratio is 1·013 when the final rate of fermentation is taken as the basis for the correction for the hydrolysis rate. The application of the more accurate correction, in which allowance is made for the increase in the hydrolysis rate due to increase in the concentration of hexosephosphate during the fermentation, would bring this to about 1·06. It is noteworthy that, whereas in Exps. 84 *a* and 85 *a*, in which glucose was employed, the evolution of CO_2 was comparatively slow and protracted, the repetition of the experiments with the addition of acetaldehyde (Exps. 84 *b* and 85 *b*) gave a much more rapid evolution of gas and also a higher ratio of CO_2 to esterified phosphate. This is almost certainly due to the smaller and more accurate correction for hydrolysis required.

On the whole then we may conclude that in the case of the dried yeasts so far studied the ratios of CO_2 to esterified phosphorus are slightly higher than unity and markedly higher than those hitherto observed with yeast juice or zymin. The origin of this small amount of extra CO_2 is at present unexplained [see Boyland, 1929].

(*b*) *Maceration extracts.* These were prepared by Lebedev's method [1912] from two samples of the dried yeast used in the foregoing experiments. The extracts in Exps. 92 and 97 were prepared from the dried yeast (English mild ale yeast) used in Exps. 93 and 94, but that used in No. 97 was prepared about 2 months after that used in No. 92. These two extracts yield ratios almost identical with those given by the dried yeast from which they were prepared, viz. 0·97 and 0·98, which would be raised to about 1·01 and 1·02

by applying the revised correction for hydrolysis. The extract employed in Exp. 99 which was made from the Dutch baker's yeast used in Exp. 98, on the other hand, has yielded a much lower ratio (0·86, 0·9 corrected) than the corresponding dried yeast (1·09, 1·11 corrected). Further experiments are necessary before any definite conclusion can be drawn as to the behaviour of maceration extract.

Kluyver and Struik [1928] have devoted much attention to this aspect of the subject and regard the ratio as indeterminate. Their experimental ratios, obtained with maceration extract, had the values 0·60, 0·71, 0·72, 0·89, 0·67, 0·71 and 0·82, most of which are below the limits we have observed. These figures are, however, not strictly comparable with ours, as the correction for the evolution of CO_2 due to change of acidity in passing from phosphate to hexosephosphate was calculated and not ascertained experimentally. They are obtained from experiments with maceration extracts using in some cases comparatively small amounts of phosphate and rather slow evolution of gas, so that the correction for hydrolysis rate is relatively larger and correspondingly less accurate than in our experiments.

It must also be remembered that the yeast employed by Kluyver and Struik was different from those we used and the difference between our results may be due to this. Further work is certainly necessary to decide this point.

(*c*) *Yeast juice.* The three experiments with yeast juice (from English mild ale yeast) (Nos. 100, 101 and 102) all gave low ratios, viz. 0·90 (0·91 corrected), 0·90 (0·92 corrected) and 0·93 (0·93 corrected), in substantial accordance with the three results quoted in our previous paper (0·87, 0·95 and 0·83).

Ratio of hexosediphosphate to hexosemonophosphate.

With dried yeast the amount of combined phosphorus in the form of hexosediphosphate is invariably considerably greater than that in the form of hexosemonophosphate, varying in our present experiments from 92·6 to 88·3 % of the total phosphorus esterified with the English mild ale yeasts, while the Dutch baker's yeast gave the lower result of 78·1 %. On the other hand the maceration extract prepared from two different dried yeasts gave very different results. Of the two extracts prepared from English mild ale yeast one, No. 92, gave 59·7 % of the total esterified phosphorus as diphosphate, and the second, No. 97, only 13·9 %. The sample prepared from a Dutch baker's yeast on the other hand (No. 99) yielded 96·9 % as diphosphate, scarcely any monophosphate being formed.

The three samples of yeast juice also gave variable results, two of them (Nos. 101 and 102) yielding 73 and 75·9 % of the total phosphorus esterified as diphosphate, whilst the third (No. 100) yielded 92·2 %.

It must further be remembered that there is an important qualitative difference between the monophosphate which is formed by dried yeast, which

is largely trehalosemonophosphate, and that formed by yeast juice and zymin, which appears to be the hexosemonophosphate studied by Robison [1922]. The nature of the monophosphate formed by maceration extract has not yet been carefully examined from this point of view.

Kluyver and Struik have also made a series of determinations of the relative amounts of mono- and di-phosphate produced by maceration extracts under various conditions of dilution and phosphate concentration. They [1928] believe that the proportion of monophosphate to diphosphate is a function of the dilution of the juice, the more dilute juice producing the greater proportion of monophosphate. Preliminary experiments made in this laboratory are not in agreement with this conclusion, which will be dealt with in a later communication.

The equation of alcoholic fermentation.

The fact which is now well established that in some instances a very large proportion of the esterified phosphorus is found in the form of hexosemonophosphate, whilst at the same time the molecular ratio of CO_2 to total esterified phosphorus is approximately unity, renders it impossible in these cases to express the result by means of Harden and Young's equation. The ratio CO_2/diphosphate in such cases is frequently very high and it is difficult to account for such large deviations from the theoretical ratio by any secondary changes such as were suggested in our previous paper.

As far as our experiments go the most definite result is that the molecular ratio of carbon dioxide to the total phosphorus esterified falls within the limits of 0·9 to 1·1, the tendency being for a low ratio with yeast juice, maceration extract and zymin, and for a high ratio with dried yeast. In other words the evolution of one molecular volume of CO_2 appears always to be accompanied by the introduction of a phosphoric group into a sugar molecule, either a mono- or a di-phosphoric ester being formed. The possibility of the occurrence of a certain amount of esterification unaccompanied by evolution of CO_2 is also not excluded, but according to our results this must be comparatively unimportant. We are yet far from understanding the actual course of events resulting in the rupture of a sugar molecule, nor can one yet say what determines the proportion of mono- and di-phosphate produced. These questions require and are receiving further investigation.

An important point which emerges from these results is that it is necessary before venturing on any generalisation to examine preparations of the most varied origins under as many modifications of conditions as possible.

Summary.

1. In fermentations of glucose or fructose in presence of phosphate carried out with dried yeast, the ratio of extra carbon dioxide evolved to phosphorus esterified tends to be slightly higher than unity.

2. With maceration extract and yeast juice this ratio tends to be somewhat lower than unity, as previously found with zymin.

3. The ratio of hexosediphosphate to hexosemonophosphate formed during the fermentation is usually high for dried yeast, but is very variable for maceration extract and yeast juice, the product in extreme cases consisting almost entirely of either the diphosphate or monophosphate.

4. The most constant ratio which we have observed is that of extra CO_2 to total P esterified, which only varies about $\pm$ 10 %, although the proportions of diphosphate and monophosphate may vary from 96 % of diphosphate to 86 % of monophosphate.

5. The equation of Harden and Young cannot be applied to those cases in which a large proportion of hexosemonophosphate is produced, whilst the ratio CO_2/total P esterified remains at about 0·9.

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XXXII. THE PREPARATION AND USE OF THE BONE PHOSPHATASE.

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The phosphatase obtained from the bones of growing animals has proved very useful in the study of the chemical composition of phosphoric esters of biochemical interest. By its aid the phosphoric acid groups may be quantitatively removed under conditions of p_H and temperature which do not involve the rupture of other linkages in the molecule such as would occur during acid hydrolysis.

For such purposes, as well as for the study of the mode of action of the enzyme, it is desirable to have stable preparations of the phosphatase, of high activity and as free as possible from blood pigments and such other impurities as would be difficult to remove from the hydrolysis products. The following is a brief account of experiments carried out with this object in view, and of the methods at present employed.

Extraction of phosphatase.

The bones of young growing rabbits are removed immediately after death, freed from muscle and connective tissue, and split longitudinally. The marrow is removed and the bones with their epiphyses are cut into smaller pieces and placed in a flask with five times their weight of water, to which a few drops of chloroform have been added. The closed flask is kept at room temperature for 7–10 days, shaken each day, and fresh chloroform added if necessary. The extract is then filtered through coarse filter paper. A second extract, much less active, may be obtained by soaking the bones in a fresh quantity of water. The progress of the extraction at 0°, room temperature, and 38° is shown in Table I. A represents the amount of hydrolysis reckoned as mg. P effected in 1 cc. 0·2 M sodium glycerophosphate by 1 cc. of the filtered extract in 1 hour at 38° at p_H 8·6. A/W is the above value divided by the weight in mg. of solid matter in 1 cc. of the extract.

Table I.

	Temperature of extraction					
	0°		Room temperature		38°	
Duration of extraction	A	A/W	A	A/W	A	A/W
18 hours	0·16	0·033	0·35	0·075	0·43	0·074
7 days	0·37	0·056	0·57	0·073	0·34	0·044
9 ,,	0·45	0·060	0·61	0·077	—	—
14 ,,	0·41	0·051	0·62	0·063	—	—
Second extraction:						
5 days	0·14	0·086	0·17	0·076	0·03	0·012

The results show that, while extraction proceeds most rapidly at 38°, some inactivation of enzyme occurs. Other comparative tests have shown that the final yield of enzyme is not greatly increased by preliminary maceration of the bones or by a series of short successive extractions. More highly active extracts are, however, obtained if the rabbits are fed for a month before death on a rickets-producing diet (McCollum's 3143) (see Table III).

Evaporation of the filtered extracts in evacuated desiccators over sulphuric acid yields 5–8 mg. of dry solid per cc., no loss of activity occurring during this process. After 7 months' storage in a dry atmosphere such residues were found to retain 60 % of the original activity of the extract.

Purification.

A very useful degree of purification may be obtained by precipitating the filtered extract with a mixture of alcohol and ether (200 cc. alcohol and 300 cc. ether for each 100 cc. extract). After shaking for a few minutes a flocculent precipitate forms, and is filtered through a Büchner funnel, washed with absolute alcohol and dried in an evacuated desiccator. If the operation is conducted expeditiously, an almost colourless powder is obtained, weighing 3–4 mg. per cc. extract. This preparation possesses the full original activity, which it retains for many weeks when stored in a desiccator. It disperses fairly well in water, and its freedom from alcohol-soluble matter renders it a suitable form of the bone phosphatase for general use.

A further degree of purification can be effected, at the expense of some loss of total activity, by extracting the above preparation with 50 % alcohol. The greater part of the enzyme passes into solution, and may be reprecipitated by a further addition of alcohol and ether. The product is now freely soluble in water and shows a greatly increased activity in relation to its weight (A/W).

Other methods of purification.

Removal of a protein by precipitation at its isoelectric point. On bringing the p_H of the aqueous extract to 5·8 a protein is precipitated, while most of the enzyme remains in solution. The latter should be brought back to the neutral point as quickly as possible after filtration. This method of purification cannot, however, be recommended owing to the risk of inactivation of the enzyme, should the necessary hydrogen ion concentration be momentarily exceeded.

Dialysis and ultrafiltration. Dialysis experiments were carried out with the object of removing the traces of inorganic phosphate which are always present in the aqueous extract, and also to investigate the possible presence of a co-enzyme. It was found that a collodion membrane, immersed for 24 hours in 95 % alcohol, was impermeable to the enzyme, which could in this way be separated from phosphates and other salts. The method of ultrafiltration in a Bechhold filter through discs of collodion of similar porosity

proved a more satisfactory procedure, the filtrate being entirely inactive and containing the whole of the phosphate. The residue on the filter may be taken up in water and precipitated with alcohol and ether as described above. The complete freedom from inorganic phosphate, while usually of no great importance, was of value in certain experiments in which it was desired to eliminate the inhibitory effect of phosphate on the activity of the enzyme.

No evidence of the presence of a co-enzyme was obtained by dialysis or ultrafiltration. A measured volume of the aqueous extract was filtered through a collodion membrane in a Bechhold filter, the residue was washed with an equal volume of water and finally dispersed in the same volume, so that 1 cc. of the residue and 2 cc. of the dialysate corresponded with 1 cc. of the original extract (Table II).

Table II.

	Inorganic P (mg. in 1 cc.)	*Activity*
1 cc. residue	0·0	0·107
2 cc. dialysate	0·052	0·0
1 cc. residue + 2 cc. dialysate	0·054	0·090
1 cc. residue + 10 cc dialysate	0·253	0·077

The activity of the residue is actually diminished by the addition of dialysate, this being due to the inhibitory effect of the inorganic phosphate thereby introduced. If a co-enzyme exists it must therefore have been still present in excess in the washed residue.

Specific adsorption. Attempts have been made to purify the enzyme by specific adsorption, using kaolin, calcium phosphate and aluminium hydroxide (type B) prepared by the method of Willstätter and Kraut [1923]. Up to the present the results have been unsatisfactory. Where adsorption has appeared to take place, as judged from the loss of activity of the solution, attempts to recover the enzyme by elution have proved unsuccessful.

Table III.

Description of preparation	*A*	*A/W*
1. 20 % aqueous extract of bones of young normal rabbits	0·61	0·077
2. ,, ,, ,, rachitic rabbits	1·15	0·120
3. Alcohol-ether precipitate of (2)	—	0·180
4. (3), extracted with 50 % alcohol, reprecipitated with alcohol-ether	—	0·570
5. Residue from ultra-filtration of aqueous extract taken up in water and precipitated with alcohol-ether	—	0·240

Table III gives the values A and A/W for various enzyme extracts and preparations purified by the above methods.

It is interesting to calculate from these values of A the amount of inorganic phosphate which could be produced in 24 hours by the phosphatase in 1 g. of bone, assuming that this phosphate is immediately precipitated and the rate of hydrolysis consequently maintained. These amounts in terms of calcium phosphate $Ca_3(PO_4)_2$, are, at p_H 8·6, 0·37 g. and 0·66 g. per g. of normal and rachitic bone respectively, while at the p_H of the blood the values

would be about 0·08 g. and 0·15 g. These figures have, of course, only limited significance, since the conditions of the test (degree of extraction of enzyme, concentration of phosphoric ester, etc.) are not those which obtain in living cartilage, but they show by their magnitude that the activity of this enzyme is quite adequate for the rôle which we have ascribed to it in the process of ossification.

Use of the enzyme as a biochemical reagent.

The following methods have been found satisfactory for the removal of phosphoric acid groups from such esters as can be hydrolysed by this enzyme.

(*a*) 1 g. of the barium salt of the phosphoric ester is placed in a small flask with 15 cc. water and 50–100 mg. of the enzyme preparation. The flask is kept at 40°; the p_H is adjusted to 8·6–8·8 and maintained approximately within that range by the addition at frequent intervals of a cold saturated solution of baryta from a burette. The use of a p_H higher than 9·0 invites the risk of rapid inactivation of the enzyme. Measurements of p_H are made on minute amounts (0·01 cc.) by means of a capillator.

The baryta required gives a rough indication of the amount of hydrolysis, but usually falls short of the equivalent of the phosphate set free. This is probably due to the precipitation of the phosphate as a mixture of $Ba_3(PO_4)_2$ and $BaHPO_4$. By this precipitation the inhibitory effect on the enzyme of an increasing concentration of inorganic phosphate [Martland and Robison, 1927] is avoided, but the rate of hydrolysis nevertheless falls, and from other experiments it is evident that the enzyme is partially adsorbed on to the precipitated phosphate. For this reason the addition of a further quantity of the enzyme preparation may be necessary in order to complete the reaction in reasonable time (4–8 hours).

Should the barium salt of the ester be sparingly soluble in water, the method is still applicable, but the flask must be frequently shaken to keep the solution saturated. When the hydrolysis approaches 100 % the enzyme, together with any barium salt left in solution, is precipitated by the addition of alcohol, and the filtered solution evaporated to dryness in an evacuated desiccator. The residue may be purified by extraction with water, evaporation of the filtered solution and re-extraction with alcohol. The product should now contain not more than a few mg. of material derived from the enzyme, this being without appreciable optical rotation or reducing power, as shown by control experiments omitting the substrate.

Should the dephosphated product be insoluble in alcohol, a different method must be employed to separate it from the enzyme material. After completion of the hydrolysis the protein may be coagulated by boiling, and the aqueous filtrate evaporated or poured into alcohol according to the properties of the dissolved substance.

(*b*) The chief disadvantage of method (*a*) lies in the alkaline reaction, which is undesirable when dealing with reducing sugars. It has been found

possible to carry out the hydrolysis at a p_H as low as 7·0 although, owing to the greatly reduced activity of the enzyme, several days may be required for the completion of the reaction. The soluble sodium salts are used and, as the liberated phosphate is not precipitated, no further addition of alkali is necessary. The isolation of the product is carried out in the same manner as described under (*a*), although the precipitation of sodium phosphate by alcohol is not so complete as that of the barium salt.

Method (*a*) as applied to esters of non-reducing sugars or sugar derivatives has given excellent results. Thus α- and β-methyl-γ-fructosides have been obtained from the corresponding derivatives of hexosediphosphoric acid [Morgan and Robison, 1928], pure crystalline trehalose has been isolated from trehalosemonophosphoric ester, which is a product of the fermentation of hexoses by dried yeast [Robison and Morgan, 1928], and gluconic acid has been obtained from the oxidation product of hexosemonophosphoric acid [Robison and King, 1929]. In all these cases a very good yield of the hydrolysis product was obtained without difficulty.

When applied to the reducing hexosephosphoric esters, both methods gave results less easy of interpretation, some of which are discussed below.

Hydrolysis of hexosediphosphoric ester.

The specific rotations of a number of the sugar products obtained by the action of the bone phosphatase on the barium and sodium salts of hexosediphosphoric acid are set forth in Table IV. The amount of ester used for each experiment was equivalent to 50–100 mg. P, the degree of hydrolysis varying from 63 to 95 %. The rotations are calculated on the amounts of sugar in the syrups, dried over sulphuric acid, as determined by the Hagedorn-Jensen method.

Table IV.

Salt used	p_H	Duration of hydrolysis	$[\alpha]_{5461}$ of sugar product
Ba	8 8	4 hours	−82°
Ba	8 8	6 ,,	60°
Na	8 8	24 ,,	- 48°
Na	8 8	24 ,,	−63°
Na	7·7	5 days	−64°
Na	7·4	4 ,,	−64°
Na	7·0	5 ,,	−76°

Hexosediphosphoric acid is believed to be a derivative of γ-fructose, which on removal of the phosphoric acid groups should change into the ordinary $\alpha\beta$-fructose. Young [1909] found, however, that the solution obtained by acid hydrolysis was less laevorotatory than a solution of pure fructose of the same reducing power. The product obtained by the action of the bone phosphatase has, likewise, a laevorotation much lower than that of fructose ($[\alpha]_{5461}$ − 111°), and the values obtained vary within rather wide limits. For the products of hydrolysis at p_H 8·6 this could be explained by assuming that the Lobry de Bruyn transformation had taken place during

the course of the reaction. It is surprising, however, that the product obtained by hydrolysis at p_H 7·0 should also have a similar low specific rotation. The possibility arises that the synthesis and hydrolysis of phosphoric esters, which must be considered as occurring simultaneously during the whole course of the experiment (even though the final equilibrium represents almost complete hydrolysis) are causatively involved in the intramolecular transformation of the hexoses. Neuberg and Leibowitz [1928] have found that the partial hydrolysis of fructose diphosphate by various phosphatases may give rise to either of the two hexosemonophosphates, which are probably derivatives of glucose and fructose respectively. The theoretical suggestion put forward by Robinson [1927], namely, that the hydrolysis of a phosphoric ester might be accompanied by a Walden inversion, is of interest in this connection, although the transformation of fructose to glucose cannot be simply explained in this way. It is hoped that more evidence will be obtained on this problem from experiments now in progress.

Summary.

Methods for the extraction and purification of the bone phosphatase and its use as a biochemical reagent are described.

The application of this method to the hydrolysis of hexosediphosphoric acid and the properties of the hexose so obtained are discussed.

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Vol. 23, p. 242: *for* —— and King (1929)
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XXXIII. INSULIN AND GLUCONEOGENESIS.

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The principal theories of the action of insulin may be shortly classified as follows.

(1) The theory of increased oxidation of carbohydrate which states that insulin brings about its typical effects by stimulating the peripheral tissues to a more rapid and greater catabolism of carbohydrate. The evidence adduced in favour of this is drawn from observations on diabetic subjects and particularly from the changes in the respiratory metabolism. All such experiments have depended on the interpretation put upon the respiratory findings. This theory demands that in the normal organism the effect of insulin be accompanied by a depletion of both liver- and muscle-glycogen, for as the peripheral carbohydrate is oxidised it is conceived that the liver delivers up its glycogen until, when both sources are exhausted, convulsions supervene and eventually the animal dies of carbohydrate deprivation.

(2) The second view is that in addition to increased oxidation there occurs also a polymerisation of carbohydrate to glycogen or to substances as yet unidentified.

In the eviscerated spinal cat, where the whole metabolism takes place in muscle, a very satisfactory balance has been struck between the sugar disappearing from the perfusion fluid on the one hand and the glycogen formation and oxygen utilisation on the other [Best *et al.*, 1926]. Cori and Cori [1928] have also been able to recover about 90 % of the sugar to be accounted for in standard rats. These authors also found that in glucose-fed rats the action of insulin was to bring about a fall in liver-glycogen and an almost equal rise in muscle-glycogen. Barbour *et al.* [1927] corroborate this finding. Cori and Cori state that under the action of insulin the peripheral need for sugar is so great that there is none available for the formation of glycogen in the liver.

(3) The third view is that originally propounded by Laufberger [1924] and more recently supported by Best *et al.* [1926] and Bissinger and Lesser [1926] as a partial explanation of the action of insulin. It states that the essential action of insulin is to inhibit the formation of glycogen from fat and protein. Laufberger strongly opposed the oxidation theory as a result of respiratory experiments on various types of animals. He also, by determining the non-sugar carbon (Restkohlenstoff) in blood, showed that the existence of a new carbohydrate complex in the blood of insulinised animals, as suggested by Macleod, was very improbable and Macleod himself has

recently admitted that the conception is unnecessary. In common with other workers Laufberger found that insulin caused the disappearance of glycogen from the liver of the normal animal, and he conceived that death from excess of insulin occurred as a result of (1) exhaustion of existing glycogen in the liver and tissues and (2) inhibition of replenishment from non-carbohydrate sources. The whole of this theory stands or falls, as Laufberger himself says, on the truth of the observation that insulin brings about an exhaustion of liver- and muscle-glycogen. He emphatically states that in no type of animal has he ever failed to find this glycogen-depleting action of insulin, whether convulsions did or did not occur.

(4) The fourth theory is that insulin produces a change in the nature of sugar so that a more readily utilisable form is produced. Various substances have been suggested but the evidence is very meagre.

It seemed to us that the fundamental matter to decide unequivocally was whether insulin did in fact diminish the liver- and muscle-glycogen in the normal animal. The diversity of the results in the literature made it probable that some underlying factor was preventing a uniformity in the findings of different workers, for there seemed no possibility of explaining the differences merely on criticisms of technique. The question arose as to whether it might not be explained by the variability in development in different species of animal. There can be no doubt that in different species the activity of such glands as the adrenal, pituitary, thyroid and sex glands is a variable factor and that this applies also to different stages in the development of the same animal. It was decided to see if uniform results could be obtained in very young animals. Young rabbits were used and comparisons made between members of the same litter. The findings with these animals have already been published [Goldblatt, 1929]. It was found that in every case after a dose of insulin (0·2–0·5 unit) sufficient to bring about convulsions in the young rabbit starved for 24 hours previously, there occurred a very large rise in liver-glycogen. Further, this increase was associated in all the cases with a blood-sugar of vanishing proportions and a muscle-glycogen not significantly affected. It was observed that the greatest rises in liver-glycogen occurred when the animals were allowed to proceed right to the onset of convulsions. In fact it was thought that with a given dose of insulin the concentration of glycogen might be a roughly linear function of the time of action.

Frank *et al.* [1927], using adult rabbits, state that if the animals be starved for 4 to 6 days their liver-glycogen does not exceed 0·2 % and they are therefore suitable for controls in comparative work with insulin. The muscle-glycogen, they state, shows no uniformity. Using 6 such animals (liver-glycogen varying from 0·05 to 0·297 %) they found that in a series of similarly starved animals treated with insulin in doses of 0·1 clinical unit, the liver-glycogen rose to 3·8 % (variations from 0·6 to 3·8 %) even when the blood-sugar fell relatively little. These authors do not state the weight or age of

their animals. In some experiments which we carried out on adult animals of 4 kg. weight the liver-glycogen after 4 days' starvation was in several cases between 2 and 3 %. In two such animals used for comparative work with insulin the control after 4 days' starvation had a liver-glycogen of 2·1 % and the other animal after injection of 0·5 clinical unit on two occasions gave a value of 2·73 %, the blood-sugar having fallen from 0·113 to 0·062 %, the total time of experiment being 3 hours 11 minutes. The difference between these results and those of Frank *et al.* may be due to the age and activity of the rabbits used, young animals losing their glycogen much more quickly on starvation than old ones. The conclusion drawn by Frank *et al.* from their experiments was that the theory of Laufberger, later supported by Dale and by Bissinger and Lesser, that the action of insulin was essentially to inhibit gluconeogenesis, is no longer tenable, Frank considering that the extra liver-glycogen is derived from body-protein. As there is no analysis of nitrogen excretion given this cannot yet be said to be proven.

There can, however, be no doubt that both in Frank's experiments and our own, using starved animals, a new formation of glycogen was taking place under the influence of insulin, and that this new glycogen was not derived from muscle-glycogen, since it has been abundantly shown that the latter cannot contribute to circulating carbohydrate [Bollman, Mann and Magath, 1925; Markowitz, 1925; Soskin, 1927].

Further consideration of the facts seems to complicate the problem. How is the fact that insulin brings about a large increase in the liver-glycogen of the fasting rabbit to be reconciled with the further fact that animals to which sugar is administered and into which insulin is injected in large doses show a progressively diminishing glycogen content in the liver? This latter fact is emphasised by Barbour *et al.* [1927], who say: "There can be no doubt that very large doses of insulin given to animals which are absorbing glucose from the alimentary canal cause more glycogen to be deposited in the muscles accompanied by an approximately corresponding decrease in the amount deposited in the liver."

This statement was made as a result of work done with standard rats, but we show below that it is also true for the young rabbit. Frank, attempting to explain this apparent anomaly, considers that, since, in order to produce convulsions in the fed animal, it is necessary to use large and unphysiological doses of insulin, there occurs a large utilisation of sugar peripherally so that the liver tends to replenish the muscle-glycogen by delivering up its own glycogen. Thus there occurs a fall in hepatic glycogen. We cannot, however, accept this view because it would necessitate in all cases the association of largely depleted liver- and muscle-glycogen with the onset of convulsions, which is not a fact [Goldblatt, 1929].

If we consider that in our young rabbits, given a dose of insulin sufficient to reduce the blood-sugar to almost zero and bring about the convulsive syndrome, there occurs a large accumulation of glycogen in the liver which

if liberated as glucose could preserve the animal's life for a considerable period, and, further, that if to such animals glucose be administered *per os* or parenterally recovery occurs, then it seems clear that, although there is a definite inhibition of glycogen release, free sugar is in quite a different position and can up to a point pass freely through the liver. This matter will be returned to later in this paper.

Experimental.

Having shown in a previous communication [Goldblatt, 1929] that the injection of insulin into young rabbits is followed invariably by a very large accumulation of glycogen in the liver, it was important to see if this occurred also in other species. For this purpose we first of all chose the rat. The very large amount of work done by Barbour *et al.* [1927] and by Cori and Cori [1928] on the metabolism of the rat made it improbable that our results would be likely to add to our knowledge of the action of insulin. These investigators have in the main obtained similar results, that insulin depletes the liver-glycogen in the starving rat and inhibits glycogen formation in the fed rat. Cori's view as a result of his work is that insulin produces so great an increase in peripheral oxidation that the liver is forced to give up its glycogen or does not form glycogen at all.

These results are so out of harmony with those we obtained with young rabbits that it is felt that there is some fundamental difference between the metabolism of these animals. In order to satisfy ourselves that the large increase in liver-glycogen could not in fact be reproduced in rats, the following experiments were carried out. The methods of analysis were the same as those detailed in our previous work.

Experiments 1 and 2 show that with doses of insulin varying from 0·2 unit to 4 units significant increases in glycogen could not be demonstrated in the liver of the rat starved for 24 hours. It is further clear that rats vary enormously in their susceptibility to insulin. Thus animal 3 in Experiment 2 developed convulsions after 0·75 unit in 1 hr. 7 mins., whilst animal 4 showed no symptoms 2 hrs. 36 mins. after 1·25 units. It is also to be remembered that the metabolism of the rat is very rapid and that during starvation they lose weight very rapidly due to the acceleration of the processes of gluconeogenesis which are necessary to replace the glycogen which they exhaust very quickly. These animals must therefore possess mechanisms, probably regulated by their endocrine system, by means of which they can rapidly mobilise carbohydrate for the needs of their muscular system. This state of affairs is in marked contrast to that found in the rabbit, which has a relatively much lower heat production. Having regard to these considerations it was to be expected that. if insulin produced a very much increased peripheral utilisation of sugar and at the same time inhibited gluconeogenesis, the rat would be a particularly suitable animal for demonstrating a rapid depletion of liver- and even muscle-glycogen.

Exp. 1. Four albino rats were starved for 24 hours. They were then treated as detailed below and determinations made of blood-sugar and liver-glycogen. The injections of insulin were made subcutaneously.

No.	Wt. g.	Blood-sugar g. per 100 cc.	Liver-glycogen g. %	Liver-glycogen mg.	Liver-glycogen per 100 g. body-wt. mg.	Remarks
1	169	0·072	0·85	38	22·5	Control—no treatment
2	129	0·069	0·69	26	20·2	0·2 unit—killed 2 hrs. 40 mins. later
3	149	0·081	0·65	28	18·8	0·4 unit—killed 2 hrs. 54 mins. later
4	106	0·051	1·00	29	27·3	0·6 unit—killed 3 hrs. later

Exp. 2. Six albino rats were starved for 24 hours and then treated as below.

No.	Wt. g.	Blood-sugar g. per 100 cc.	Liver-glycogen g. %	Liver-glycogen mg.	Liver-glycogen per 100 g. body-wt. mg.	Remarks
1	184	0·100	0·59	34	18·5	Control
2	119	0·018	0·67	27	22·7	2.13 p.m. 0·5 unit 3.10 ,, Killed
3	110	—	0·82	28	25·6	2.18 ,, 0·75 unit 3.25 ,, Convulsions 3.30 ,, Killed
4	138	0·033	0·95	33	23·9	2.24 ,, 1·25 units 5.0 ,, Killed
5	188	—	1·11	52	27·7	2.30 ,, 1·5 units 5.51 ,, 2·0 units 7.12 ,, Killed Hypoglycaemic during 40 mins.
6	199	0·020	0·74	45	22·6	2.35 ,, 2 units 5.12 ,, 2 units 5.35 ,, Convulsions and killed

Dale and his co-workers have indeed drawn attention to this point. But our results cannot be brought into line with any theory which attributes an inhibition of gluconeogenesis to insulin, for whatever may have occurred in the peripheral tissues there certainly did not occur any exhaustion of liver-glycogen, which leaves no doubt in our mind that formation of carbohydrate from non-carbohydrate sources was proceeding quite adequately in spite of the active effect of the insulin.

Exp. 3. Four young rats were taken without previous starvation. One was kept as a control and the other three received subcutaneous injections of 1, 2, 3 units (clinical) of insulin. The latter animals were allowed to die in convulsions. The control was killed after the others had died. No food was allowed during the experiment.

Carcase signifies what remained after decapitation and removal of the abdominal viscera.

No.	Wt. g.	Blood-sugar g. per 100 cc.	Liver-glycogen g. %	Liver-glycogen mg.	Carcase-glycogen g. %	Carcase-glycogen mg.	Total glycogen per 100 g. body-wt.	Remarks
1	57	0·030	0·35	8	0·21	47	96	3 units—convulsions 29 mins. after injection, lasted 37 mins., when animal died
2	53	"0"	0·53	11	0·12	33	83	2 units—convulsions 22 mins. after injection, lasted 50 mins., when animal died
3	49	"0"	0·42	10	0·21	46	114	1 unit—convulsions 24 mins. after injection, lasted 52 mins., when animal died
4	61	0·113	0·49	11	0·24	64	123	Control—killed after the others had died

The first noteworthy point in Exp. 3 is the exceedingly low values for glycogen in these small animals, even unstarved, which gives some idea of the necessity for frequent feeding in order to spare the tissues. The enormous doses of insulin used and the very severe convulsions which supervened were clearly not able to bring about an exhaustion of either liver- or carcase-glycogen. Definitely less glycogen was found in the insulinised animals than in the control, but this did not apply to any extent in the liver.

Exp. 4. Four albino rats were starved for 24 hours and were then given a mixture of 3 g. flour and 5 g. glucose worked up into a soft mass with a little water. They all ate the mass readily and after 3 hrs. had consumed almost all of it. Injection of insulin took place immediately on presenting the food mixture.

No.	Wt. g.	Liver-glycogen g. %	Liver-glycogen mg.	Liver-glycogen per 100 g. body-wt. g.	Remarks
1	132	4·63	182	1·38	Control –killed 4 hrs. after presenting food
2	163	2·86	142	0·87	0·2 unit insulin—killed 4 hrs. after presenting food
3	152	4·27	177	1·16	0·4 unit insulin—killed 4 hrs. 10 mins. after presenting food
4	138	4·95	187	1·36	0·6 unit insulin—killed 4 hrs. 30 mins. after presenting food

Unfortunately in this experiment the blood-sugar values could not be determined. The figures are, however, of some interest in view of the findings of Barbour *et al.* that up to 3 hours after the injection of moderate doses of insulin in fed rats there occurs a retardation of glycogen deposition in the liver. Of course, no more can be concluded from this experiment than that it is at least possible to have rats which, when fed with plenty of carbohydrate and treated with doses of insulin varying from 1 to 5 units per kg., can lay down quantities of glycogen of the same order. Although this to some extent opposes the findings of Barbour *et al.* [1927] and of Cori and Cori [1928] we do not deny the principle involved in their findings, for similar results to theirs could be obtained using young rabbits, as shown in the next experiment.

Exp. 5. Six young rabbits from the same litter, 7 weeks old, were starved for 24 hours before the experiment. 20 cc. of 50 % glucose solution were given by mouth by means of a tube and syringe. Insulin was injected immediately afterwards and the animals were killed 3 hrs. later.

No.	Wt. g.	Blood-sugar g. per 100 cc.	Liver-glycogen g. %	Liver-glycogen Total g.	Remarks
1	667	0·154	0·55	0·10	Control—no treatment
2	774	0·224	5·00	1·54	10 g. glucose *per os*
3	714	0·197	3·82	0·84	,, ,, ,, —2 units insulin
4	760	0·159	3·56	0·82	,, ,, ,, —4 ,,
5	737	0·090	2·20	0·59	,, ,, ,, —6 ,,
6	725	0·058	2·15	0·51	,, ,, ,, —8 ,,

None of the animals in this experiment showed any symptoms. The progressively lower glycogen in the liver with increasing doses of insulin was very striking, particularly when one considers that it is possible to obtain

values of liver-glycogen up to 4 % in insulinised animals given no food at all (see below).

Examination of the intestinal contents of all the animals which had been given glucose showed that there were still large quantities of reducing sugar available. In spite of this the animals were approaching hypoglycaemia which, as is well known, could have been averted by giving more sugar. This puzzling circumstance recalls the fact that after the administration of sugar to the normal animal the temporary rise in blood-sugar is followed by a marked fall, even to hypoglycaemic levels, at a time when there are still large quantities in the gut and the respiratory metabolism has returned to normal levels. This phenomenon is usually explained as being the result of a stimulus to storage in the liver arising from the raised blood-sugar and the degree of saturation of the muscles with carbohydrate. This conception clearly cannot be applied to such an experiment as No. 5.

If absorption from the gut is at the same rate in every case then there seems to be no alternative but to regard the lower liver-glycogens as due to the excessive utilisation in the muscles brought about by the very large doses of insulin. But we feel that this question of absorption is precisely the difficulty. This matter is being investigated and no final decision can as yet be given.

The question of controlled absorption arises again if we consider the changes in blood-sugar after successive doses of glucose. The following experiment illustrates this.

Exp. 6. The blood-sugar changes in normal men following 4 successive doses of 50 g. glucose each. The first was given 3 hrs. after the morning meal.

	Time	Blood-sugar g. per 100 cc.	Remarks
Subject 1:			
	12.00	0·102	12.05 p.m. 50 g. glucose
	12.30	0·150	
	1.00	0·125	1.00 ,, ,,
	1.30	0·135	
	2.00	0·125	2.00 ,, ,,
	2.30	0·109	
	3.00	0·125	3.00 ,, ,,
	4.00	0·117	
Subject 2:			
	11.40	0·113	11.45 a.m. 50 g. glucose
	12.15	0·122	
	12.45	0·122	
	1.15	0·104	1.30 p.m. ,,
	2.00	0·122	
	2.30	0·125	2.50 ,, ,,
	3.05	0·113	
	3.20	0·129	
	3.35	0·100	3.50 ,, ,,
	4.15	0·117	

The first point of interest in these figures is that one cannot force up the blood-sugar of the normal subject by successive doses of sugar: all that is possible is to prevent the fall to the rather low levels which commonly follow the administration of a single dose. Examination of these tables will show that at three points the administration of the sugar was followed in the first

half-hour by a fall in blood-sugar, to be succeeded by a return to the original or a rather lower level. Such a fall has never been observed by us following a single dose of sugar. It appears, therefore, that there exists a very delicate mechanism by means of which the processes of absorption and discharge of sugar from the liver are regulated to the needs of the peripheral tissues. Thus, in starvation, when the immediate needs of the tissues are being supplied by gluconeogenesis, the administration of sugar is followed by blood-sugar values far in excess of the normal which are sustained at high levels for a very prolonged time. The respiratory metabolism in such cases shows much less rise in R.Q. and what rise does occur takes place during the fall in blood-sugar. The same delay in the rise of R.Q. also occurs in the ordinary post-absorptive state. Accepting the usual interpretation of the R.Q. this would mean that there is a lag in the oxidative mechanism after the presentation of the sugar to the tissues, which would suggest that a certain concentration of carbohydrate must be reached in the tissues before the process of oxidation is commenced or the process of gluconeogenesis is diminished.

Now the height of the blood-sugar at any time is proportional to the ratio $\frac{\text{rate of liberation from the liver}}{\text{rate of peripheral utilisation}}$ and the normal organism seems at all times to tend to readjust this ratio to an equilibrium value which keeps the blood-sugar in the region of 0·100 g. per 100 cc. Permanent disturbance in either or both the terms of this ratio must result in a pathological condition.

The possible number of variations of this ratio is nine, according as we have unchanged, increased and diminished activity of the two factors. Every case of ortho-, hyper-, and hypo-glycaemia must be covered by these variations.

Now after the administration in the post-absorptive state of a dose of glucose *per os* there is at first a rapid delivery of sugar to the muscles and a temporarily almost exclusively carbohydrate oxidation. There then seems to occur a fall in sugar oxidation and then the blood-sugar falls. There must hence be a fall in both terms of the above ratio until, when the mass of available carbohydrate reaches a certain level, gluconeogenesis is brought into play in order to keep the ratio as near the equilibrium point as possible.

Now, since an animal can go into insulin convulsions with quantities of sugar in the gut, it seems unavoidable to consider that the concentration of the intestinal sugar has fallen to a level at which the rate of absorption is inadequate to combat the hypoglycaemic action of the insulin, although the absolute amount may be quite sufficient. The effect of dilution can easily be shown by taking 50 g. glucose dissolved in a very large volume of water, say a litre, when the rise in blood-sugar will be found to be very slight indeed.

But the fact, as seen in Exp. 5, that progressively increasing large doses of insulin acting for the same time are associated with progressively less formation of glycogen from free sugar, indicates that the passage of sugar

through the liver is so rapid that a large quantity of glucose has passed through the liver before the dilution effect comes into play. This is not the same as saying that the animal can deplete its liver-glycogen when under the influence of insulin, which is the opinion of Frank and others. For, as we have seen in young animals, and Chaikoff [1925] found in adults, death from insulin convulsions may occur in the presence of high liver-glycogen values. What is suggested is that free sugar can, under the necessities brought about by large doses of insulin, pass rapidly through the liver, the amount remaining available for formation of glycogen being correspondingly less. But such glycogen as is formed, we conceive, is fixed so that it is not available for the relief of the severe condition which later arises.

In the next experiment further evidence was sought for the view that insulin inhibits glycogen release.

Exp. 7. The writer was the subject of experiment. In order to establish a state in which the process of gluconeogenesis was actively proceeding, a preliminary starvation of 40 hours was undergone. Insulin was then injected subcutaneously and when hypoglycaemia was well established glucose was taken. As evidence of gluconeogenesis we may take the degree of ketosis, for a large body of evidence exists that the ketonic acids arise as products of this process. These substances were estimated by a method described by the writer for small quantities of urine [Goldblatt, 1925].

Time	Urine cc.	Rothera reaction	Acetoacetic acid mg. per hr.	β-Hydroxy-butyric acid mg. per hr.	Blood-sugar g. per 100 cc.
11.00 a.m.	33·0	+ +	6·8	7·4	—
11.30 ,,	13·0	+ +	—	—	—
11.52 ,,	—	—	—	—	0·077
11.54 ,,	8·0	+ +	9·3	4·8	—
11.57 ,,	10 units insulin hypodermically				0·062
12.00 ,,	—	—	—	—	—
12.30 p.m.	19·0	+ +	17·4	39·6	0·065
12.45 ,,	50 g. glucose in 200 cc. water taken				0·054
12.47 ,,	10·0	+ +	—	—	—
1.00 ,,	—	—	—	—	—
1.15 ,,	10·0	+ +	11·6	13·6	0·082
1.30 ,,	8·5	+ +	—	—	—
1.45 ,,	9·5	+	6·8	7·6	0·109
2.00 ,,	10·0	—	0	0	—
2.15 ,,	15·0	—	0	0	0·122
2.30 ,,	9·5	—	—	—	—
2.45 ,,	14·0	—	—	—	0·122
3.00 ,,	10·0	—	0	0	—
3.15 ,,	—	—	—	—	0·069
3.30 ,,	17·0	—	0	0	—
3.45 ,,	—	—	—	—	0·045
4.00 ,,	15·5	—	0	0	—
4.15 ,,	—	—	—	—	0·027

At this stage a strong hypoglycaemic reaction was experienced. Glucose, bread and tea were administered and recovery took place in about an hour. At 5.30 there was glycosuria and a positive Rothera reaction and at 5.45 the blood-sugar was 0·137 g. per 100 cc. In interpreting these findings the following points must be noted.

(*a*) After this period of starvation there is ample glycogen in the liver and tissues but gluconeogenesis is proceeding as indicated by the ketosis.

Since the latter can be completely abolished by giving glucose or any other good glycogen former, we consider that it is the concentration of glycogen in the liver which determines the degree of gluconeogenesis.

(*b*) When the excretion of the ketonic acids falls to zero we consider that the glycogen in the liver is at an optimum for the normal supply of carbohydrate to the tissues.

The first point we note in this experiment is the rapidity of action of the insulin, pre-hypoglycaemia being definitely experienced in 33 mins., when the blood-sugar had fallen to 0·054 g. per 100 cc. 50 g. glucose in 200 cc. water having been taken, a severe hypoglycaemic attack occurred $3\frac{1}{2}$ hours later. Examination of the blood-sugar values shows that although the maximum value was only 0·122, the absolute rise above the level attained when the sugar was taken was 0·068 g. per 100 cc., which is not significantly different from the increase usually obtained after such a dose of sugar without insulin. Thus the rate of passage of sugar through the liver was up to this point adequate to counteract the effect of the peripheral utilisation. This maximum point was associated with the disappearance of the ketosis and, as we believe, with a concentration of glycogen in the liver sufficient to inhibit gluconeogenesis.

The phenomena now become different, for the blood-sugar values fall steadily until very low levels are attained (0·027 g. per 100 cc.) and still the ketosis remains absent. No urine could be collected between 4.15 and 5.30 p.m. as the subject was prostrate; but the nitroprusside reaction was distinctly positive at the latter time and at 5.45 p.m., *i.e.* when the action of the insulin had worn off. Examination of the values for the excretion of acetoacetic acid and β-hydroxybutyric acid shows that there occurred a large rise in the amounts of these substances after the injection of the insulin. Similar results have been recorded by Collip [1923], who observed the production of ketone bodies in hypoglycaemic rabbits, and by Burn and Ling [1928], who report very large increases in the ketosis of rats adapted to a fatty diet, following the injection of insulin. We have not been able to confirm these latter findings but the results in the above experiment are suggestive.

Now, although it is probable from Exp. 5 that there was less glycogen in the liver than there would have been had no insulin been injected, there could be no doubt that even when a dangerous hypoglycaemia had been reached there was an amount present high enough to prevent gluconeogenesis. This, we consider, corroborates our view that the outstanding effect of insulin in the intact animal is to inhibit the release of such glycogen as is already present in the liver.

In the following experiments a relationship was sought between the formation of new liver-glycogen, the dose of insulin and its time of action in the starving young rabbit.

Exp. 8. In this experiment the time of action was kept constant and the dose varied. Five young rabbits, 6 weeks old, from the same litter were starved for 24 hrs. and were killed in all cases about 2 hrs. after the injection of the insulin, whether convulsions had or had not supervened.

No.	Wt. g.	Blood-sugar g. per 100 cc.	Liver glycogen g. %	Liver glycogen Total mg.	Liver glycogen Increase over control mg.	Remarks
1	493	0.141	0.82	139	-	Control
2	399	0.058	1.71	297	158	0.25 unit—slight convulsive attack. Killed 2 hrs. 10 mins. after insulin
3	472	0.062	1.03	167	28	0.50 unit—no symptoms. Killed after 2 hrs. 9 mins.
4	590	0.058	0.99	185	46	0.75 unit—no symptoms. Killed after 2 hrs. 7 mins.
5	682	0.058	0.69	167	28	1.0 unit—no symptoms. Killed after 2 hrs. 5 mins.

It has been found in these young animals that the glycogen content of the liver after 24 hours' starvation is higher in the winter (when the experiments recorded in this paper were carried out) than in the summer. Further, they seem to be more resistant to insulin in the winter than in the summer, as judged by the rapidity of onset of convulsions after a given dose. The results of the above experiment show that the degree of gluconeogenesis in the liver is not so much a function of the dose of insulin as of the rapidity of approach to the convulsive state. The only animal which showed a rise in the liver-glycogen which we can regard as considerable was No. 2, which was the only one that exhibited a convulsive attack and it had received the smallest dose of insulin. We must, therefore, consider that the large new formation of glycogen is a response to some urgent necessity arising from the intensity of action of the insulin. In the next experiment the dose of insulin was kept constant and the time of action varied. Estimations of muscle (gastrocnemii)-glycogen were also carried out in each case. Determinations of so-called "free sugar" were also made in liver and muscle. This was done as follows. About 5 g. of tissue were dropped into a weighed stoppered centrifuge tube of about 100 cc. capacity containing 20 cc. absolute alcohol, which had been kept in ice. After weighing, the tissue was minced under the alcohol as finely as possible with sharp scissors, which were washed clean with 20 cc. 70 % alcohol. The contents were now shaken periodically and kept in ice for 2 hours and then centrifuged. The clear supernatant fluid was then pipetted off and run into a graduated 100 cc. flask. The entire operation was then repeated and the supernatant fluid again added to the flask. The alcohol was now removed on the water-bath and the residual fluid, which was of a yellowish colour, worked up for estimation of sugar as follows. 20 cc. of 15 % sodium sulphate and 3 drops of glacial acetic acid were added to the flask and, after warming on the water-bath, 1 cc. of colloidal ferric hydroxide was added. The flask was cooled and the liquid made up to 100 cc. and filtered. Reducing power was estimated in 20 cc. of the filtrate by the method of von Issekutz and von Both [1927]. In regard to this method

it must be mentioned that when dealing with extracts of tissue allowance must be made for iodine-binding substances by a preliminary titration in the cold. Although there can be no question of the figures thus obtained being entirely due to sugar, it was thought that any considerable change in tissue-sugar would show itself by this method.

Exp. 9. Seven young female rabbits from the same litter, 6 weeks old, were starved for 24 hrs. Two were taken as controls, four were given 0·5 unit of insulin subcutaneously and killed at different times afterwards. One was given sufficient insulin to put it into convulsions and then killed.

			Liver			Muscle		
		Blood-sugar	Glycogen		"Free sugar"			
No.	Wt. g.	g. per 100 cc.	g. %	Total mg.	-Total mg.	Glycogen g. %	"Free sugar" g. %	Dose of insulin and time of action
1	546	0	0·81	142	127	0·28	--	Control
2	563	0·122	0·62	155	—	0·29	0·27	,,
3	535	0·035	1·28	225	—	0·28	0·25	0·5 unit — 30 mins.
4	538	0·056	0·78	152	66	0·32	- -	0·5 ,, — 60 ,,
5	535	"0"	1·19	195	—	0·32	0·31	0·5 ,, -- 97 ,,
6	492	0·053	3·62	761	90	0·30	- -	0·5 ,, — 145 ,,
7	587	0·035	4·43	1159	—	0·23	0·28	0·5 ,, —3.30 p.m. 0·5 ,, —6.25 ,, 0·5 ,, 8.00 ,, 2·0 units—8.15 ,, Hypoglycaemia and killed—9.00 p.m.

It was again seen that the control values for liver-glycogen were much higher than those found in similar animals in summer. The muscle-glycogen was, however, of the same order as always found in these animals.

The first point elicited from this experiment was that, although there occurred marked rises in liver-glycogen in animals 3 and 5, animal 4 showed no change, which we think shows that the amassing of liver-glycogen is not merely a function of the time of action of the same dose. The time of action is, however, clearly of great importance as is seen from the findings with animal 6, which, without any manifest symptoms, was found to have a liver-glycogen of 3·62 % and a total of 761 mg., *i.e.* 5 times the control value. It seems as if the process of gluconeogenesis were suddenly stimulated to a very much increased activity by an emergency created by the insulin at its point of maximum effectivity. No. 7 illustrates very strikingly the progressive nature of this glycogen deposition in the liver and demonstrates beyond doubt that insulin, in these circumstances, so far from "clearing out" the glycogen, is bringing about a very rapid gluconeogenesis.

The values for muscle-glycogen were remarkably uniform and left no doubt that whatever carbohydrate was being used peripherally was being adequately replenished from new sources as the insulin continued its action. The "free sugar" in the liver was very suggestive of an inhibition of glycogenolysis, for the ratio of this to glycogen fell from 0·9 in the first control to 0·43 in No. 4 and then to 0·12 in No. 6. In the muscles, however, no such striking changes were found. These results suggest that whilst insulin may

inhibit the change from glycogen to glucose in the liver, it does not do so in the muscles.

From the last two experiments we consider that insulin can stimulate the process of gluconeogenesis in the liver, and that this effect is most obvious when the animal is at the stage of convulsions.

SUMMARY.

1. Evidence is presented that insulin can stimulate the new formation of glycogen from endogenous sources.

2. It is considered that the theory that insulin inhibits gluconeogenesis is untenable.

I am indebted to Dr H. R. Youngman for assistance in the carrying out of the last two experiments.

It is a pleasure to express my gratitude to Prof. Hugh MacLean for his unfailing advice and encouragement.

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XXXIV. THE HYDROGEN ION CONCENTRATION AND THE CALCIUM AND PHOSPHORUS CONTENT OF THE FAECES OF RACHITIC CHILDREN.

By THIRZA REDMAN.

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(*Received March 14th, 1929.*)

In a previous paper [Redman, 1928] an attempt was made to correlate the p_H of the faeces of rachitic children with their clinical condition, the children being kept in hospital on a special diet, but different groups undergoing different methods of treatment, and their clinical condition being controlled by X-ray photographs. In the present paper another aspect of the same work is described.

The investigation consisted in the determination of the p_H of fresh samples of faeces and the estimation of the calcium and phosphorus in the same samples when dried. The p_H values were determined by means of the quinhydrone electrode.

Calcium was estimated in the following manner. A suitable quantity of dried faeces was burnt to a grey ash in a vitreosil crucible. The ash was extracted with hot dilute HCl and well washed with hot water. The filtrate was made just alkaline to methyl red with ammonia and then just acid with acetic acid; it was then boiled, precipitated with boiling ammonium oxalate solution and left overnight. The precipitate was filtered off, washed, dissolved in hot, dilute H_2SO_4 and titrated with standard permanganate solution.

For the estimation of phosphorus a portion of dried faeces was digested in a round-bottomed flask with a mixture of conc. H_2SO_4 and conc. HNO_3 till all the nitric oxide fumes were boiled off and oxidation was complete.

The liquid was cooled, diluted with water and made slightly alkaline with ammonia, then just acid with nitric acid and a volume of 50 % ammonium nitrate added. After heating to 70° the solution was precipitated with ammonium molybdate mixture, well shaken, cooled and filtered by suction. The precipitate was washed with 10 % HNO_3, then with 2 % NH_4NO_3 and lastly with distilled water. It was then washed back into the flask, dissolved in a known volume of $N/2$ NaOH, boiled for 20 minutes, cooled and titrated with $N/2$ H_2SO_4, using phenolphthalein as indicator. 1-2 cc. of acid were added in excess and the solution boiled again for 15 minutes. It was then cooled again and accurately titrated with $N/2$ NaOH.

Curves were then plotted to show the relationship between the p_H and the percentages of calcium and phosphorus in the faeces at different periods of time under the same conditions of diet, but under different treatments, until healing of the rickets was established.

The children were kept on the following diet:

Milk	Mince
Cream	Minced chicken
Cocoa, made with milk	Bacon gravy
Raw egg beaten in milk	Bread
Blancmange	Rusks
Custard	Butter
Beef tea or chicken broth	Oranges

Two younger children (Nos. 48 and 49) did not receive mince, chicken or cocoa.

The different treatments were as follows:

1. Cod-liver oil and malt together with
   (*a*) artificial sunlight (tungsten-cored carbon arc),
   (*b*) mercury vapour lamp.
2. "Ostelin."
3. Irradiated ergosterol.
4. "Radio-malt."
5. Irradiated cholesterol.

The routine method of dealing with the faeces was described in the previous paper.

Curves have been plotted from one or two representative cases from each group of children and from one control child on the same diet, but not suffering from rickets. Figs. 1 to 7 show the curves for rachitic children and Fig. 8 shows the curve for the normal child: the ordinates on the left refer to "percentages," those on the right refer to p_H, while the abscissae refer to the periods of time in days.

Figs. 1 and 2 (cod-liver oil) show a pronounced fall in the output of calcium in both cases 34 and 36 as treatment proceeds and healing occurs, but a rise in No. 34 towards the time of complete cure.

A tremendous drop occurred simultaneously in the amount of phosphorus in case 34 but was not so marked in case 36. The phosphorus curve crossed the calcium curve about the 25th day in case 34, but unfortunately data were not obtained for case 36, in which the two curves were approaching each other towards the 15th day.

The calcium curve swings in a corresponding manner to the p_H curve in the later part of case 34, and practically all the time in case 36.

In Fig. 3 (case 38, "ostelin"), calcium output fell to a minimum about the 23rd day. The phosphorus curve did not reach its minimum till later, on the 29th day.

No correlation between p_H and either calcium or phosphorus was apparent.

Fig. 4 (case 40, irradiated ergosterol) is remarkable in that the calcium values are almost all lower than those obtained for phosphorus; also for the fact that, on this treatment, cases took a much longer time to heal.

In Fig. 5 (case 44, "radio-malt") great variations occur in the calcium but

not nearly so marked in the phosphorus output. Again, the phosphorus curve crosses the calcium curve as healing occurs and then diverges from it. The

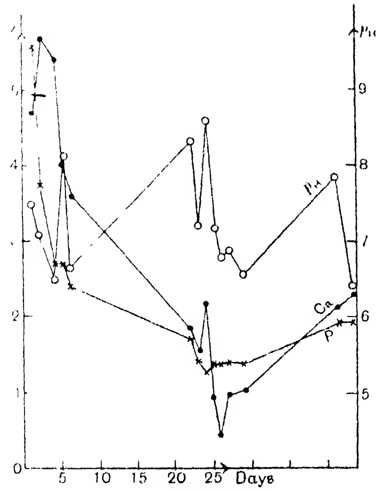

Fig. 1. *Case* 34. *Treatment.* Cod-liver oil and A.S *X-ray.* Cured 51 days.

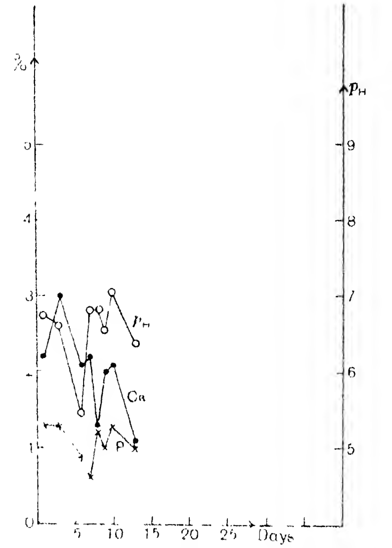

Fig. 2. *Case* 36. *Treatment.* Cod-liver oil and Hg vapour lamp. *X-ray.* Cured 38 days.

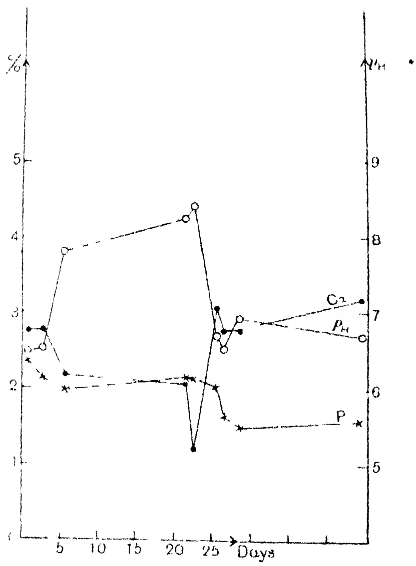

Fig. 3. *Case* 38. *Treatment.* "Ostelin." *X-ray.* Cured 62 days.

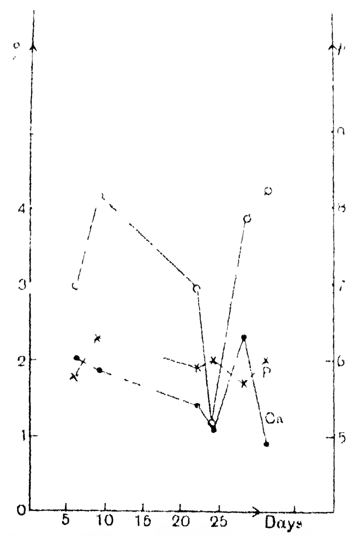

Fig. 4. *Case* 40. *Treatment.* Irradiated ergosterol. *X-ray.* Nearly cured 63 days.

•———• Calcium ×———× Phosphorus o———o p_H

p_H and calcium curves swing together for the greater part of the time. Clinically, the case improved rapidly in general condition and intelligence.

Figs. 6 and 7 (cases 48 and 49, irradiated cholesterol) show tremendous variations in calcium over the first 15 days but greater output of phosphorus

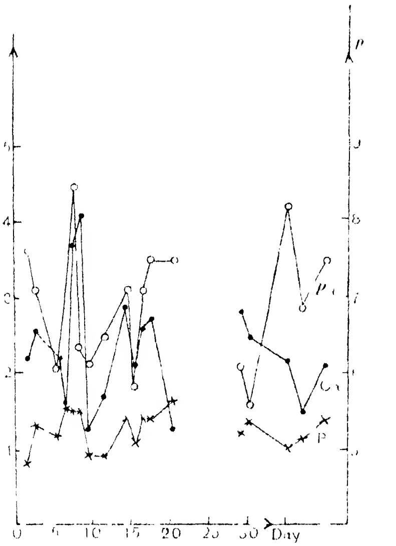

Fig. 5. *Case* 44. *Treatment.* "Radio-malt." *X-ray.* Nearly cured 38 days.

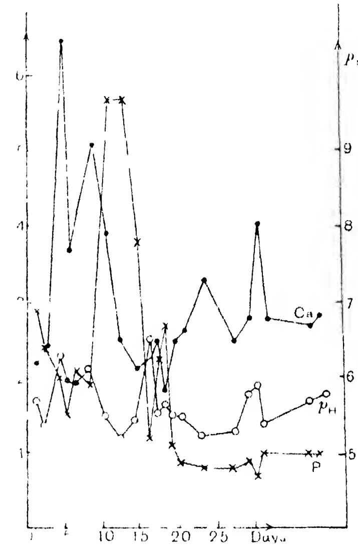

Fig. 6. *Case* 48. *Treatment.* Irradiated cholesterol. *X ray.* Cured 35 days.

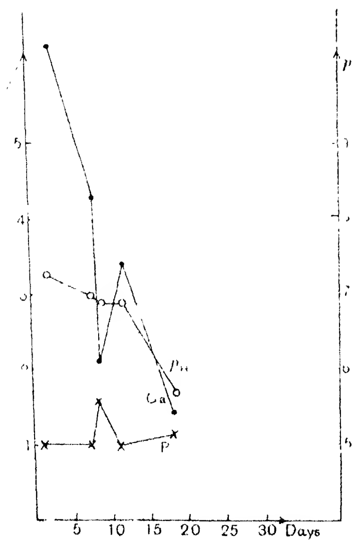

Fig. 7. *Case* 49. *Treatment.* Irradiated cholesterol. *X-ray.* Nearly cured 36 days.

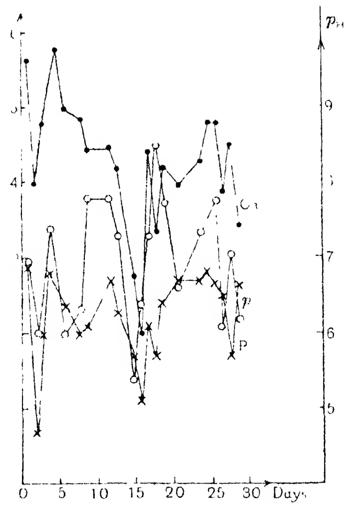

Fig. 8. *Case* 47. *Normal child.*

•——• Calcium ×——× Phosphorus o——o p_H

in No. 48 than No. 49. The improvement in the clinical condition of both these children was almost dramatic after a few days' treatment. It is notice-

able that in case 48 the phosphorus curve crossed the calcium curve before the 10th day and rose to a great height before settling down. At this period very loose stools occurred and the quantity of irradiated cholesterol administered had to be reduced. This is interesting in view of recent work pointing to the ill-effects produced by over-dosage with vitamins in concentrated form. Towards the 18th day the phosphorus curve crossed the calcium curve again, then fell and remained consistently low as complete cure was established.

In case 49 the phosphorus values were low throughout and the child made excellent progress till cured. The p_H values were all below 7 in case 48 and nearly all below 7 in case 49, and can be fairly well correlated with calcium values but not with those for phosphorus.

Fig. 8 (case 47, normal child) shows variations in both calcium and phosphorus but no crossing of the calcium curve by the phosphorus curve, both curves rising and falling fairly well together and with a certain amount of correlation with the p_H curve.

Summary.

The results suggest a certain degree of correlation between p_H and the percentage of calcium in the faeces of rachitic children.

The best clinical results were obtained by the use of irradiated cholesterol and it is noteworthy that p_H values were practically all below 7, which may indicate some confirmation of the work of Zucker and Matzner [1924], and of Bacharach and Jephcott [1926], on rachitic rats in which it was shown that when healing occurred the reaction of the faeces became acid and remained so. On the other hand, the cases in question (Nos. 48 and 49) were on a diet from which the mince and chicken, given to the rest of the children, were excluded and the p_H of the faeces may have been influenced thereby.

Of other cases, healing appeared to be more rapid when the percentage of phosphorus was consistently low, *e.g.* No. 36 on cod-liver oil and mercury vapour lamp treatment, and No. 44 on "radio-malt." In the former case the p_H was less than 7 on all occasions but one.

I wish to express my gratitude to Col. Macdiarmid (Medical Superintendent) and to the Staff of Alder Hey Hospital for all their help during many months of work on cases under their care, to Prof. Heilbron and Dr Kamm of the University of Liverpool for the preparation of many batches of irradiated cholesterol and ergosterol and to Prof. Beattie, Liverpool University, in whose department the work was carried out during the tenure of the Virol Research Scholarship in Clinical Pathology.

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XXXV. HYPERVITAMINOSIS AND VITAMIN BALANCE.

PART II. THE SPECIFICITY OF VITAMIN D IN IRRADIATED ERGOSTEROL POISONING.

PART III. THE PATHOLOGY OF HYPERVITAMINOSIS D.

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(Report to the Medical Research Council.)

(Received February 28th, 1929.)

PART II. THE SPECIFICITY OF VITAMIN D IN IRRADIATED ERGOSTEROL POISONING.

IN the course of earlier papers Harris and Moore [1928, 1, 2] confirmed the toxic action of irradiated ergosterol in excess and concluded that "a weight of evidence pointed to the toxic action of excess of vitamin D *per se*." Recently it has been suggested, however, that the poisonous effects are not due to the vitamin, but are confined to certain preparations in which poisonous by-products have been allowed to develop. While we have not wished to deny the possibility of such additional toxic by-products we continue in our belief that vitamin D is the main and essential toxic ingredient present in irradiated ergosterol. Additional evidence in support of this conclusion is described in this communication.

Doubts have been raised on several occasions as to the reality of the parallelism between vitamin content and toxicity. Thus after we had completed in June, 1928, a series of observations upon solid irradiated ergosterol, prepared by activation in alcoholic solutions, it was suggested to us by Messrs British Drug Houses, Ltd., who had kindly supplied the specimen, that toxicity was peculiar to samples which had been prepared in this way. Experiments in their laboratories had in fact independently confirmed the toxic action of ergosterol when irradiated in alcohol, but toxic effects had never been observed in ergosterol activated by the routine method employed for "radiostol," a commercial preparation manufactured without the use of alcohol. In the same direction Dixon and Hoyle report [1928] that using ergosterol irradiated in oil by Messrs British Drug Houses, Ltd., they had been unable to confirm the alleged toxicity of irradiated ergosterol ("vigantol") as described by Kreitmair and Moll [1928]. In their conclusions they throw doubt on the assumption of hypervitaminosis, suggest instead that some independent toxic factor must have been present in the alleged toxic specimen of irradiated ergosterol, and aver that there is "evidence to show that when

irradiated in alcohol the product is toxic." Recently statements have appeared in the medical press that "radiostol" is irradiated in such a way as to give freedom from toxic by-products.

As a result of experiments with medicinal "radiostol," however, we have been able to demonstrate that such toxic properties are by no means restricted to samples of ergosterol activated by the alcohol method. Weight for weight, in fact, the irradiated ergosterol of "radiostol" has been found to be as poisonous in excess as the solid product used in our first experiment. We can only suppose that in previous experiments in feeding excess of these sources an insufficient allowance of the preparation was administered, or that administration was not continued over a sufficient period to allow the development of the more severe symptoms of hypervitaminosis.

Experimental.

1. *Non-toxicity of irradiated ergosterol in which vitamin D has been destroyed by prolonged over-irradiation.*

The result of this test was alluded to in our earlier paper, but experimental details have not yet been given.

The over-irradiated ergosterol was prepared from the same specimen of irradiated ergosterol that was fed to a control group of animals, and proved toxic to them. It was exposed in a thin film in a porcelain dish for 24 hours at a distance of 30 cm.[1]

Three groups of rats were taken weighing about 90 g., and from one litter. The same basal diet was fed to all animals, but supplemented in the case of the first group with non-irradiated ergosterol, in that of the second group with the irradiated[2] ergosterol, and in the third with over-irradiated ergosterol, at the same concentration in each case, 0·1 % (*i.e.* 10 mg. of ergosterol in a 10 g. daily ration). The basal diet was the same as that previously described [Harris and Moore, 1928, 2, p. 1463], and all animals in each group received in addition 0·75 cc. of marmite and 2 drops of cod-liver oil *per diem*.

Those animals receiving irradiated ergosterol lost weight rapidly, and showed the symptoms previously described, including loss of appetite, marked cachexia, and a 20 % diminution in heart rate, and died after 20 to 34 days: *post mortem* examination showed the symptoms typical of hypervitaminosis D to be described later, notably enormous deposits of calcium salts in various sites in the body. Those having over-irradiated ergosterol, on the other hand, were after 35 days growing at the same rate as the normal controls receiving non-irradiated ergosterol; there were no signs of ill-health, heart rates were

[1] The specimens of irradiated, over-irradiated, and non-irradiated ergosterol were assayed for their vitamin D activities (by examination of rib junctions and by X-ray pictures of the long bones) and it was shown that the over-irradiated ergosterol had been reduced to at least 1/1000 of the original activity of the irradiated ergosterol.

[2] Irradiated by the method previously described [Harris and Moore, 1928, 2].

normal, and on being killed and autopsied no abnormalities, macroscopic or microscopic, were seen.

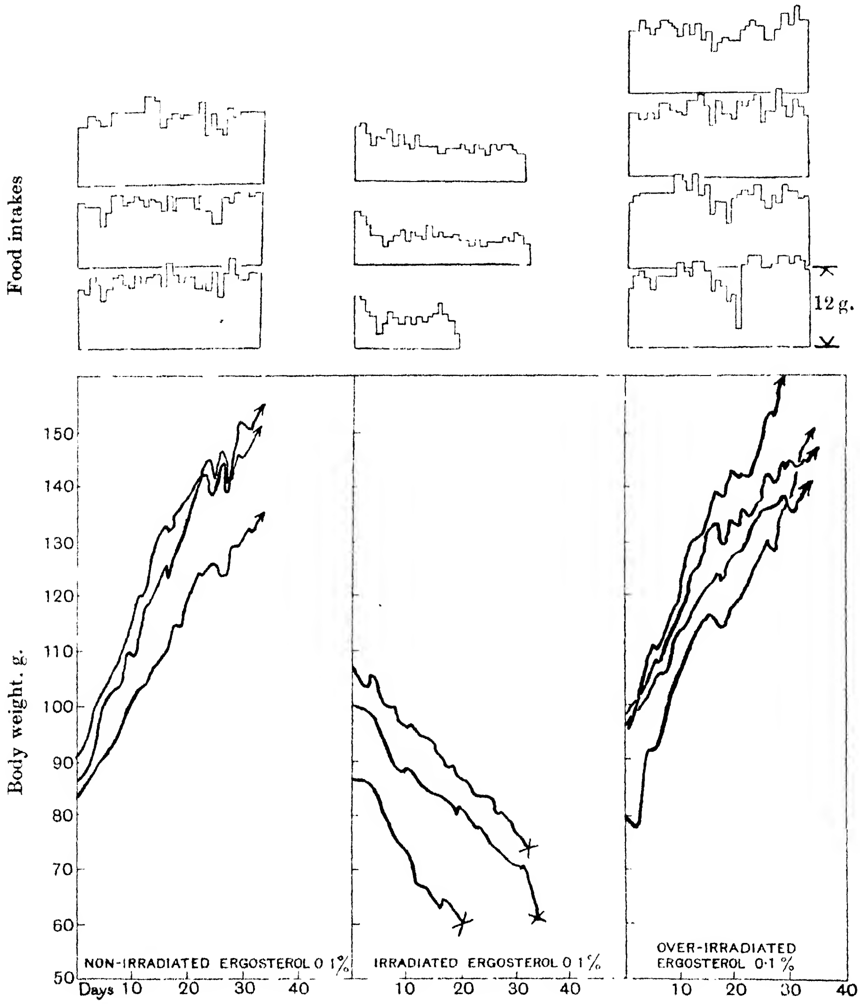

Fig. 1. Loss of toxicity on destruction of vitamin D by over-irradiation.

2. *Comparison of toxicity of oil-irradiated ergosterol ("radiostol") with alcohol-irradiated ergosterol.*

Rats of about 40 g. from one litter were divided into three groups. One group was given a diet containing 0·025 % of alcohol-irradiated ergosterol. The second group was given a similar diet containing approximately the same number of units of vitamin D, but in this case provided in the form of "radiostol" (oil-irradiated). The third group served as normal controls receiving basal diets of the same percentage composition but from which the ergosterol

only was omitted. (All three diets contained: purified caseinogen 20 %, rice starch 55 %, salt mixture 5 %, and fat 20 %, the last ingredient being in the case of the controls plain arachis oil, in the case of the alcohol-irradiated ergosterol group arachis oil containing 0·125 % of accurately assayed ergosterol, and in the case of the "radiostol" group "radiostol" itself. All diets were supplemented with marmite, 0·75 cc., and cod-liver oil. 2 drops per animal *per diem.*)

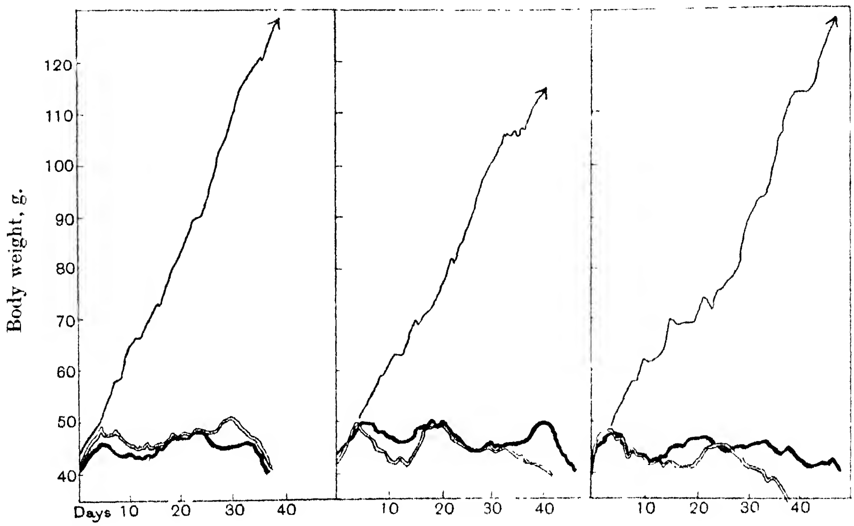

Fig. 2. Comparison of toxicity of oil- and alcohol-irradiated specimens of ergosterol.
Alcohol-irradiated ergosterol (0·025 %).
"Radiostol" (oil-irradiated) at same vitamin D level.
Controls without excessive vitamin D.

Average daily food intakes: alcohol-irradiated, 5, 5, 5 g. (moist weight); oil-irradiated, 5, 5, 5 g. (moist weight); controls, 6–12 g., increasing during expt.

The results showed that there was a remarkable identity in the degree of toxicity of the irradiated ergosterol at a given level of intake, irrespective of the form in which it was administered, either as "radiostol" or "solid" (alcohol-irradiated) ergosterol. The close parallelism of weight curves is noteworthy (see Fig. 2); it was impossible to distinguish one group of animals from the other by appearance, and both showed similar but slight changes in heart rate, much less marked in this case, of course, than when ergosterol was given at four times the concentration, as for example in Exp. 1 above (Fig. 1). Death occurred after the same lapse of time with "radiostol" as with alcohol-irradiated ergosterol. *Post mortem* both groups were found to be very emaciated, there was diminution in size of spleen, atrophy of thymus, and great deposits of calcium in kidneys, aorta, heart musculature and other sites including calculi in bladder in several instances. No abnormalities could be detected in the control "arachis oil" group.

3. *Ergosterol irradiated in absence of solvent.*

Through the kindness of Prof. J. C. Drummond we were fortunate in being provided with a specimen of ergosterol of more than average purity. This we irradiated in a thin film in an atmosphere of nitrogen in a quartz flask for 240 mins. at a distance of 20 cm. from a mercury-vapour lamp. The ergosterol thus irradiated was then incorporated in the "synthetic" diet previously described (at 0·1 % level) and fed to a pair of rats weighing 40 g. In spite of the fact that our procedure in the absence of any solvent was probably inadequate to produce maximal antirachitic activity—insufficient material being available in this instance for an exact assay—the animals resuming growth at almost normal rate after 14 days; nevertheless, when they were killed after 28 days histological examination by Mr J. R. M. Innes revealed the typical extensive calcium deposits in kidney, aorta, etc. referred to above.

Discussion.

The reality of hypervitaminosis D.

The above chemical evidence is uniformly in favour of our belief that vitamin D *per se* is toxic when administered in large excess. We have shown that toxicity is developed in ergosterol by irradiation, but that it is destroyed with the vitamin by prolonged over-irradiation. Toxicity is absent again from ergosterol resinised in such a way as to cause no formation of vitamin D, although such treatment might be expected to produce impurities similar to the by-products of irradiation [Harris and Moore, 1928. 2]. It would be a remarkable coincidence to find that the hypothetical toxic by-product followed the presence of vitamin D so closely and so invariably as this.

By proving the toxicity of preparations obtained by irradiation in oil, the supposed confinement of toxicity to "alcohol-irradiated" samples is definitely ruled out. Toxicity has been found to run parallel with vitamin D content no matter what mode of preparation has been adopted. Moreover, this toxicity is made manifest in the same ill-effects and in the same *post mortem* findings which in themselves are very suggestive.

The possibility of additional toxic substances being present in certain samples of irradiated ergosterol. however, obviously cannot be excluded.

It has been stated by Underhill [1928] that by using ergosterol irradiated in a non-volatile solvent results were obtained which confirmed our own with alcohol-irradiated ergosterol. Nevertheless he considers the deleterious effects to be not entirely due to vitamin D itself, since ergosterol irradiated under different conditions "and subjected to further manipulation may have much greater deleterious results." It is impossible to deny the existence of such hypothetical toxic by-products but we consider that the above chemical evidence considered in conjunction with evidence of the marked disturbances in calcium and phosphorus metabolism which are the foremost characteristic of

ALCOHOL-IRRADIATED ERGOSTEROL

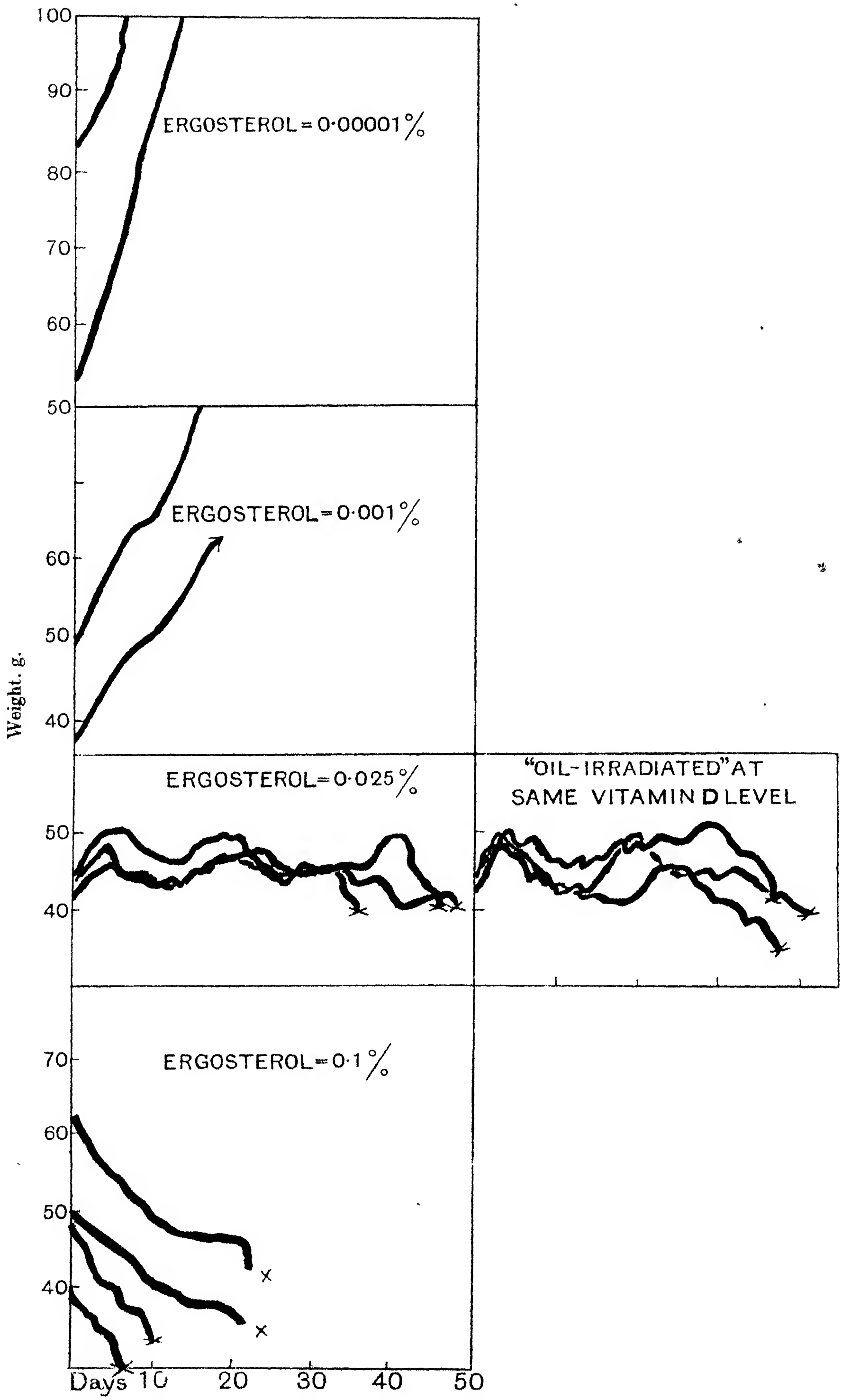

Fig. 3. Toxicity of irradiated ergosterol at different vitamin D levels. (See Part III.)

irradiated ergosterol poisoning (see Part III) amply justify our belief that the conditions described constitute a genuine state of hypervitaminosis D.

In Part III possible reasons are considered for the failure of several investigators to obtain symptoms of hypervitaminosis.

Part III. The pathology of hypervitaminosis D.

As to the pathology associated with excessive vitamin D intake, two opposing conclusions have so far been put forward.

On the one side, Pfannenstiel [1927] had found that rapid loss of weight and death were caused in his experimental animals, and Kreitmair and Moll [1928] in confirming this described the presence of massive deposits of calcium in many sites in the body (in the blood-vessels, heart musculature, kidneys, lungs and other organs).

On the other side, Dixon and Hoyle [1928], who failed to confirm the German work and were led to throw doubt on the assumption of hypervitaminosis, found no definite symptoms (except occasional haematuria) and when the animal was killed for *post mortem* examination they found in the organs neither pathological changes in the microscopical sections nor deposits of calcium, nor pathological lesions—except those associated with urinary calculi, which were found in every case (except one) at the highest level of vitamin D feeding[1].

Our own experimental animals, on all of the various high vitamin D diets already described, have at *post mortem* examination invariably shown the condition described by Kreitmair and Moll. Urinary calculi have frequently been evident as well. Several new aspects of hypervitaminosis have also come to light, and we have been surprised to note the remarkably rapid cures, with considerable disappearance of calcium deposits, which occurred when the excessive vitamin D was withdrawn from the diet.

Experimental.

(1) p_H *of faeces.*

On certain diets the absence of vitamin D is known to cause an increased alkalinity of the faeces, and when the vitamin is again given the faeces revert, temporarily at least, to a more acid reaction [Zucker and Matzner, 1923; Jephcott and Bacharach, 1926; Redman, Willimott and Wokes, 1927; Yoder, 1927; Grayzel and Miller, 1928]. Moreover artificial acidification of the contents of the gut [Irving and Ferguson, 1925], or the addition of acid to the food [Zucker *et al.*, 1922] can alleviate the effects of vitamin D deficiency (*i.e.* low blood-Ca or -P or both). Since, then, vitamin D deficiency tends to result in diminished acidity of faeces, it seemed of interest to determine whether under some circumstances vitamin D excess might be accompanied

[1] Kreitmair and Moll had also found that in hypervitaminosis the spleen shrank to a small fraction of its normal size: Dixon and Hoyle obtained, in one group of rats, 20 % diminution in weight only.

by an increased acidity, just as we have found in other ways too that the results of vitamin excess are in fact the opposite to those of vitamin deficiency, *e.g.* blood-phosphate [Harris and Stewart, 1929] or blood-calcium, or degree of calcification.

The p_H determinations were made in the usual manner [Jephcott and Bacharach, 1926] by Miss E. Alchorne and Mr A. L. Bacharach, to whom we are greatly indebted. When the basal diet used was in a sense an unpromising one for our purpose—viz. one on which the reverse change (from acid to alkaline on a vitamin D-deficient diet) is not readily, or at all, attained—no decisive increase in faecal acidity was obtained. It is true that we were

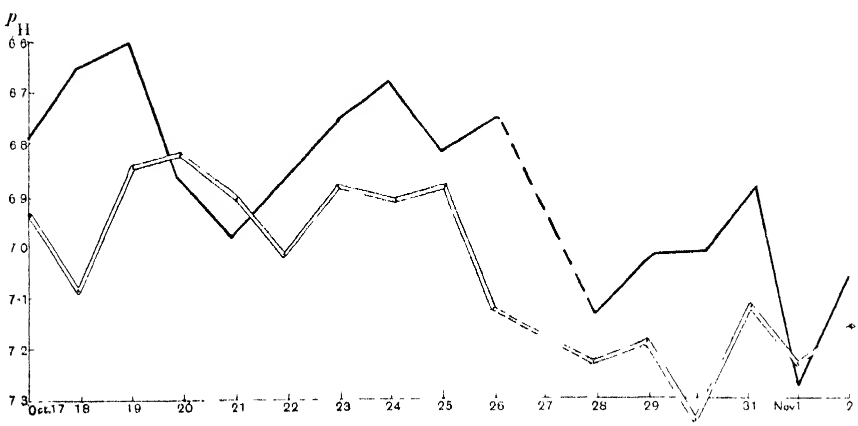

Fig. 4. p_H of faeces. Each curve is the average for three animals. *Single line*=excessive irradiated ergosterol (0·1 %). *Double line*=non-irradiated ergosterol. Normal Ca—P ratio in diet.

No clear separation into two distinct p_H zones could be effected. Animals showed great fluctuation in faecal p_H from day to day. The mean p_H increased during the course of the experiment. (Out of a total of 15 daily sets of readings, the lowest p_H recorded was on 14 occasions from an animal in the "excess vitamin" group, and the highest p_H recorded was on 11 occasions from an animal in the "non-irradiated" group.)

successful in showing that there was some decrease in the average daily faecal p_H of the animals receiving excessive irradiated ergosterol over the controls receiving excessive non-irradiated ergosterol, yet individual animals in the irradiated group not infrequently had a faecal p_H just as high or even higher than individual animals in the non-irradiated group.

Further work is in progress using a diet on which the change of reaction with absence of vitamin D occurs more readily (modified Pappenheimer, low P—high Ca) also with high P—low Ca diets.

(2) *Influence upon the heart beat.*

In the course of a separate electrocardiographic investigation, now in progress in collaboration with Dr A. N. Drury, we have found that the production of hypervitaminosis is invariably accompanied by a progressive diminution in rate of heart beat. A fall of 20 % or more was observed at the level of

0·1 % of irradiated ergosterol, but much less at 0·025 % ("radiostol" and alcohol-irradiated ergosterol).

(3) *Other symptoms.*

The remarkably rapid loss in weight (comparable with that in starvation), frequent diarrhoea, lowered food intake, and marked cachexia, greatly raised blood-phosphate [Harris and Stewart, 1929], and urine literally saturated with calcium salts, have been referred to elsewhere.

(4) Post mortem *findings.*

We are deeply indebted to Prof. J. B. Buxton and his staff for carrying out *post mortem* examinations.

(*a*) *Controls.* Animals which were killed after receiving the various control diets, containing (1) non-irradiated ergosterol, (2) non-irradiated ergosterol resinised by heating, (3) over-irradiated ergosterol or (4) oily solvents alone, were well nourished and showed no abnormalities, macroscopically, or in microscopic section, except for the occasional presence in the kidney of separate minute calcareous spots, such as are not infrequently observed in the normal rat.

(*b*) *Irradiated ergosterol* ("*radiostol*," *etc.*). Closely concordant findings were obtained in all instances, and as a typical example, those resulting in cases of death from excessive "radiostol" may be cited[1].

(1) *Macroscopic examination* showed that the animal was very emaciated, with an entire lack of subcutaneous fat.

Spleen. One-half normal size (with atrophy of Malpighian bodies), regenerating upon cure.

Thymus. Atrophied and less than one-quarter normal size (in some cases it had practically disappeared), regenerating with cure.

Bladder. Contained (*e.g.*) 5 calculi[2] as large as pins' heads and gritty material[3].

Calcareous deposits. Tangible deposits, visible to the naked eye, in kidneys (which were beset with white areas), in heart-muscle (whitish bands), in pyloric end of stomach, aorta (walls thickened and sclerosed), colon, etc.

This abundant calcification has been previously observed by Kreitmair and Moll, but the extensive changes in the thymus and the condition of the urine do not appear to have been previously noticed.

(2) *Microscopic examination.* Specimens were cut in each experimental group from the heart, aorta, kidneys, stomach, duodenum, spleen, thymus, etc. Detailed histological findings are presented elsewhere, but the main conclusions regarding calcification are typified in the case of animals on 0·025 % "radiostol," alluded to above.

[1] Level of intake equivalent to 0·025 % of highly active solid irradiated ergosterol in the diet.

[2] Identified chemically.

[3] In rabbits the urine in the bladder had become cloudy with excessive calcium salts, as shown chemically.

Aorta. Extensive calcification in the tunica media, causing the elastic lamina to become separate and intima to be raised from underlying structure.

Kidneys. Extensive areas of calcification in medulla, cortex and pelvis; and casts in the ducts of Bellini.

Heart musculature. The small arteries of the muscle definitely thickened and calcified, in some cases forming a complete ring. Some of the bigger branches of coronary arteries show the whole arterial coat to be converted into one calcified mass obscuring all structural detail.

Cartilaginous tissues. Calcification had occurred.

Duodenum. Calcification in reticular tissues.

Small intestine. Calcification in progress.

The calcification of cartilage appears to have escaped notice hitherto.

(5) *Effects of age, and degree of vitamin excess.*

Age. In the first place, as might be anticipated, we find that there is always a tendency for an older animal to survive longer on any given vitamin excess than for a younger one.

Table I.

0·1 % irradiated ergosterol.

Initial weight of animal, g.	40	49	50	51	53	64	86	100	106
Days elapsing before death	6	10	7	24	10	25	20	34	32

Similarly, disturbances in levels of blood-phosphorus and -calcium appear to be less easily produced in the older animal [Harris and Stewart, 1929; Hess, Weinstock and Rivkin, 1928].

Degree of excess. More important are the effects of differences in the degrees of the excess of vitamin D fed. Now we find that irradiated ergosterol gives rise to definite hypervitaminosis at a level of 0·025 % of the diet no less than at 0·1 %. But at the latter concentration there occurs a remarkably rapid loss of weight, at the rate of some 1 or 2 g. *per diem*, which begins immediately, on the first day of the experiment. On the other hand, at the slightly lower concentration of 0·025 % there is a cessation of growth but little or no loss of weight, and there is a considerable lengthening of the period which elapses before the animal finally succumbs to the hypervitaminosis, compared with that on the fourfold concentration (see Fig. 3, Part II). The *post mortem* findings differ somewhat in the two cases. The more prolonged hypervitaminosis (on the lower concentration of irradiated ergosterol) tends to result, it is found, in greater accumulation of calcareous deposits in the form of urinary calculi. When, however, death occurs more swiftly following rapid loss of weight (on the slightly higher concentration of irradiated ergosterol) calcium deposits may be seen as before as white bands or spotted areas in the heart muscle, kidney, colon, etc., but there appears to have been—not unnaturally—less opportunity for the growth of large calculi.

DISCUSSION.

Nature of hypervitaminosis.

It would appear that there are two threshold values for vitamin D, a minimum and a maximum. Below the minimum, there result deficiencies in blood-phosphorus, or -calcium (or both), and a consequent deficient calcification: the primary factor is a diminished retention of these two elements. Above the maximum threshold value on the other hand, there result excessively high blood-phosphorus or -calcium (or both) and a consequent excessive calcification. To some extent, as we have seen, the blood-calcium in hypervitaminosis is prevented from reaching too high a level by increased precipitation of calcium deposits and by excretion.

Several other aspects of hypervitaminosis are still quite obscure. As to the meaning of the atropic changes in the spleen and thymus there is as yet little evidence, but the reverse changes have been reported in rickets and we are examining the problem also in relation to the extirpation of these organs. The observed rapid fall in weight, with the suggestion of increased general metabolism, must also call for further enquiry, in the process of which we may reasonably expect some light to be thrown on the mode of action of vitamin D in the body.

Comparison of divergent results of different workers.

Our findings confirm those of Kreitmair and Moll [1928] that hypervitaminosis D is associated with gross disturbances in calcium metabolism. It remains to consider why other workers feeding large overdoses have failed to observe similar disturbances.

In the experiments of Dixon and Hoyle [1928] ergosterol which had been irradiated in oil was administered to rats at levels of 1, 2, 11 and 17 mg. *per diem* without giving rise to the symptoms of hypervitaminosis described by Kreitmair and Moll. When the 17 mg. level was reached, however, they did observe urinary calculi in their experimental animals. Their results also show that, while at levels of 1, 2 and 11 mg. there was good growth rate, at 17 mg. growth was poor, in fact had practically ceased. It is interesting to speculate whether the toxic threshold of vitamin D dosage may not have just been reached, and whether had they increased the vitamin D activity yet further, another four times at least, definite symptoms of hypervitaminosis might not have appeared. In a similar way Rosenheim and Webster [1927], who mentioned that they had found irradiated ergosterol to be harmless to a rat at 10,000 times the minimum effective dose, had no doubt not quite reached that concentration of vitamin D which is definitely toxic—100,000 times the minimum effective dose was needed in our experiments to cause a rapid loss of weight.

The absence of marked symptoms of hypervitaminosis in experiments on the human adult may probably be explained on similar grounds. By feeding

irradiated ergosterol at a dosage of 8 mg. for 21 days Havard and Hoyle [1928] observed neither injurious effects nor any change in blood-inorganic phosphorus or blood-serum. Although Kroetz [1927] reported that by feeding 18–30 mg. of the material great changes in mineral balance[1] were induced, he also found that such doses were apparently harmless. One is tempted to think that if it were possible for a still larger quantity to be taken, and over a sufficiently prolonged period, some rise in calcium or phosphorus must ensue. In the adult rabbit we find that the first rise in blood-phosphorus on the toxic dose of 10 mg. per day may not occur until after a fortnight [Harris and Stewart, 1929]. Calculating on the admittedly rough basis of relative body weights[2] from the lethal dose for the rat (1–10 mg.) and for the guinea-pig (about 40 mg.) the equivalent for a human of 10 stone would be some 10 g. *per diem*, and it may well be that the amounts consumed by these investigators were insufficient to cause any definite rise, particularly when one recalls that the adult is less susceptible to these changes than the young animal.

Since Part I was published, confirmation of the phenomenon of hypervitaminosis D has appeared in the independent work of Collazo, Rubino and Varela [1929], who name the condition "vitaminismus." We also learn privately of confirmatory evidence obtained in other laboratories.

Summary.

Part II.

1. Evidence is presented in support of the belief that excessive vitamin D *per se* induces a condition of specific hypervitaminosis, apart from possible separate (but undemonstrated) ill-effects due to poisonous by-products, if any.

2. "Radiostol" (activated in oil) and irradiated ergosterol (activated in alcohol) when fed at the same vitamin D levels produced hypervitaminosis of the same degree of severity. Ergosterol irradiated in absence of any solvent also produced specific ill-effects.

3. Fuller details are given of tests showing that the toxicity of irradiated ergosterol disappears concurrently with the destruction of its vitamin D by over-irradiation, and that non-irradiated ergosterol and ergosterol which has been resinised without the production of vitamin D are both non-toxic.

Part III.

4. *Post mortem* examination after death from hypervitaminosis D showed not only enormous deposits of calcium in the heart musculature, kidneys, and other organs as noted by Kreitmair and Moll, but often in addition the presence of urinary calculi, which were reported in the conflicting result of

[1] The renal excretion of P, Cl and water was found to be much increased, together with a lowering of blood-P and some acidosis. The effect is certainly surprising since it is the opposite of that produced by administration of vitamin D to the rachitic infant.

[2] The lethal dose varies appreciably from one species to another.

Dixon and Hoyle to occur in the absence of any such generalised hypervitaminosis.

5. Features of experimental hypervitaminosis in rats and rabbits, now observed for the first time, include—in addition to a remarkably high blood-phosphate—a tendency towards diminished p_H of faeces, diminished heart rate and at death atrophy of the thymus.

6. It would appear that there are low and high threshold values for vitamin D, below and above which there are caused deficient and excessive values respectively of blood-phosphorus and/or blood-calcium and resulting calcification. Atrophy of the thymus and spleen may be contrasted with the enlargement or hypertrophy often seen in clinical rickets.

The feeding of the experimental animals has been carefully attended to by Mr A. Ward.

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XXXVI. FURTHER STUDIES OF THE CHEMICAL NATURE OF VITAMIN A.

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Introduction.

In a previous paper on this subject [Drummond, Channon and Coward, 1925], the claim of Takahashi and his colleagues [1922, 1923, 1925] to have isolated vitamin A from cod-liver oil was criticised. It was shown that the fraction to which they gave the name "biosterin" was probably a complex mixture containing, in addition to the vitamin, unidentified unsaturated alcohols and possibly hydrocarbons of the squalene type. The investigation we are now reporting represents an extension of our earlier work, and the results provide conclusive proof that "biosterin" consists to a very large extent of substances other than vitamin A.

The earlier experience in this laboratory led us to believe that the considerable difficulties encountered in separating the constituents of the unsaponifiable fraction of cod-liver oil might to some extent be lessened if a much larger amount of material were available for fractionation than had formerly been employed. Accordingly plans were made for preparing the unsaponifiable fraction from a very large quantity of cod-liver oil. Through the generosity of Mr R. B. Job and Mr W. A. Munn of St Johns, Newfoundland, we were provided with 125 gallons of a high quality medicinal cod-liver oil. Actually the hopes based on the possession of the large amount of unsaponifiable material from this oil were not realised, for peculiar difficulties encountered in its examination made it advisable to change the line of attack.

It was decided to make a comparative study of the vitamin fractions from several different sources in order to ascertain whether a correlation between the vitamin content and any chemical characteristic could be traced. For this part of the investigation four materials were selected, namely, the unsaponifiable fractions from (*a*) cod-liver oil, (*b*) sheep-liver fat, (*c*) Greenland shark-liver oil, (*d*) Japanese shark-liver oil.

Although we have failed by this means to throw any clear light on the chemical nature of vitamin A, we believe we have established that the active substance is present in liver-oil concentrates in amounts so minute that direct attempts at its isolation by the ordinary chemical methods are of little use.

Experimental.

A. *Cod-liver oil.*

A large quantity of medicinal cod-liver oil from Newfoundland was saponified on a semi-technical scale in the laboratories of the British Drug Houses. We are greatly indebted to Mr F. H. Carr for his advice and assistance in carrying out this part of the work. The product yielded by this treatment was re-saponified in our laboratory with alcoholic KOH, and the extracted unsaponifiable fraction was again hydrolysed, this time with sodium ethoxide. All these operations were carried out with special precautions against oxidation.

The yield was about 0·7 % of the oil, and the product was a clean orange-coloured crystalline wax. The vitamin A colour value[1] was 13·3.

As in previous work, the first step was to remove the greater part of the cholesterol by crystallisation from methyl alcohol or light petroleum. In this manner several hundred grams of a dark red oil (I) with the following properties were obtained:

Cholesterol content	13·35 %
Refractive index n_D^{40}	1·5022
Iodine value[2]	161
Vitamin A colour value	20

Our previous studies had led us to believe that this material contains in addition to cholesterol a number of unsaturated alcohols which might possibly be separated by the preparation of suitable derivatives. Weidemann [1926] attempted to separate the alcohols present in the corresponding fraction of Greenland shark-liver oil by means of the phthalates, but the record of his experience did not encourage us.

After a considerable amount of exploratory work along these lines we concluded that it would prove a matter of very great difficulty to effect any sort of separation of the constituents of the vitamin fraction of cod-liver oil by this means. No satisfactory crystalline derivatives were obtained, even

[1] Vitamin A colour values. It is not easy to find a satisfactory manner in which to express the relative powers of concentrates to yield the blue colour reaction of Rosenheim and Drummond. The most satisfactory estimations of the colour produced by arsenic or antimony trichloride under the conditions described by Carr and Price [1926] are made between 5 and 10 blue units. Throughout this paper the colour values of concentrates are expressed in the form of the reciprocal of the percentage concentration in grams which gives 10 blue units when 0·2 cc. of the chloroform solution is treated with 2 cc. of a 30 % solution of antimony trichloride in chloroform, and the colour measured after 30 seconds in a tube of 1 cm. diameter. We have relied almost entirely on the colour reaction for detecting and estimating vitamin A throughout this study. In an investigation which is shortly to be reported we have submitted the colour test to a further critical examination, and, as a result, believe it to be a more accurate method of estimating vitamin A than the biological method.

[2] The majority of the iodine values given in this paper were determined by using the method of Dam [1924].

when the substituted bromo- and nitro-phthalates were prepared, and finally we abandoned this line of attack.

There remained the possibility that with the larger amounts of material at our disposal a more satisfactory separation by distillation in high vacuum might be obtained. In some respects the distillations reported in the earlier work were unsatisfactory. In the first place they were conducted at relatively high pressures (1 to 2 mm.), and secondly, although it was shown by colour reaction and by feeding test that vitamin A had survived the process, no very clear evidence was obtained as to the amount of loss that had occurred. This was due to the unreliability of the biological test as a quantitative measure of vitamin A, and to the fact that the colour reaction was at that time (1924-5) carried out with sulphuric acid, and was therefore quite unsuitable for quantitative assay.

In view of the great expense we could not in the present study entertain the idea of removing completely by precipitation with digitonin the cholesterol present in our raw material, and an attempt was made to distil it without this treatment. This was done with some reluctance as we had always found more decomposition to occur when fractions were distilled without removal of the precipitable sterol.

The distillation was carried out in an apparatus so designed that it could be filled or swept through rapidly with a current of purified nitrogen. Nitrogen for this purpose was "scrubbed" satisfactorily by allowing the gas to escape from the cylinder through a fine-pored filter candle immersed in an alkaline solution of sodium hydrosulphite. It emerges in the form of a cloud of very small bubbles which do not coalesce, and a very satisfactory removal of the traces of oxygen which are normally present in cylinder nitrogen is attained, particularly if the column of hydrosulphite is high, and the gas is stored for a few hours in contact with the solution before use. The surfaces of the hydrosulphite in the washing vessel and the reservoir are protected by a layer of liquid paraffin.

The side arm of the distillation flask was fitted with a coil of nichrome wire, both inside and outside the receiver, so that electrical heating could be applied if there was any tendency for the distillate to thicken or solidify.

The maintenance of low pressures was assisted by having in the system a silica tube of adsorbent charcoal cooled in liquid air.

Distillation of red oil (I) from unsaponifiable matter of cod-liver oil.

As a preliminary experiment, 44 g. were distilled under what were regarded as satisfactory conditions, namely, uniformly low pressure of 0·01 mm. and a relatively small temperature difference (20–25°) between the metal-bath and the point of distillation. The course of the distillation is represented by curve *A* in Fig. 1, p. 288. It is unsatisfactory in that there is not much

evidence of fractionation. Either the material was a very complex mixture or decomposition had occurred.

The following fractions were, however, taken:

Fraction No.	Weight g.	Appearance	Temp.	Iodine value	Cholesterol %	n^{40}	$\alpha^{20°}_{5461}$	Vitamin value
1	3·0	Pale brown liquid	80°–170°	119	0·0	1·4813	+0·8	0·14
2	1·7	Clear brown liquid, few crystals	170°–190°	115	2·12	1·4857	+0·5	0·86
3	4·8	Clear brown liquid	190°–222°	134	2·83	1·4872	−0·2	2·0
4	9·3	,,	222°–245°	176	10·80	1·4886	−2·7	0·5
5	6·8	Opaque brown semi-solid fat	245°–260°	176	20·65	1·4935	−6·95	0·22

The original material had a vitamin value of 20. If the recovery of vitamin be calculated from the weights of the fractions and their respective vitamin values it will be seen that it is as low as 2 %. Towards the end of the distillation the temperature was tending to rise rather rapidly, and signs of some decomposition were evident. A relatively large proportion of undistilled material (18 g.) remained behind in the flask in the form of a dark brown tar, which had an iodine value of 137, and contained 19·9 % of cholesterol.

All the fractions possessed the pungent terpene-like odour which suggested that decomposition had occurred. In our previous work [Drummond, Channon and Coward, 1925], we noticed that this odour was less pronounced when material was distilled after complete removal of the sterol. The estimations of cholesterol in the above fractions did not suggest that cholesterol itself decomposed, as the recovery was practically quantitative.

In spite of the very large loss of vitamin an attempt was made to study the material further. In view of the fact that no apparent separation had been effected by the distillation, the fractions that gave the best colour reactions (2, 3 and 4) were combined for further examination.

Some of this mixture was treated with digitonin to remove the precipitable sterol. It yielded a dark reddish brown oil with an iodine value of 138. As no crystalline products or derivatives could be isolated from it, an attempt was made to reduce it with hydrogen in the presence of a suitable catalyst in the hope that solid material would result. The oil proved very resistant to hydrogenation at room temperature when using as catalyst platinum prepared by the method of Feulgen [1921]. The hydrogen was absorbed very slowly, and it was found necessary to renew the catalyst several times. The hydrogenated product was a pale, brown-coloured, semi-solid fat possessing an iodine value of 48, and containing a small amount of glistening crystalline material. The solid portion was separated and recrystallised from methyl alcohol. It separated in the form of shimmering plates. Insufficient was obtained for satisfactory purification, but the best preparation melted at 54–56°. No other crystalline substance was isolated from the hydrogenated material. The colour reaction for vitamin A was not given by the reduced fraction.

The smoothly ascending distillation curve obtained in the distillation of the cod-liver oil unsaponifiable fractions, together with the unexpectedly great loss of vitamin A, led us to believe that more general decomposition had occurred during the heating than we had imagined. It was possible that decomposition products were responsible for the apparently rapid poisoning of the catalyst, and for the consequent slow uptake of hydrogen by the distilled fractions.

Accordingly, an attempt was made to reduce some of the undistilled red oil (I) after removing the sterol precipitable by digitonin. This material proved, however, to be equally resistant to reduction by hydrogen in the presence of platinum. In one experiment 10 g. dissolved in 200 cc. of absolute alcohol were treated with hydrogen in the presence of 0·1 g. of platinum. Hydrogen was admitted at a pressure of about an atmosphere and a half, and the reaction vessel rapidly shaken. The uptake of hydrogen was very slow, and several additions of fresh catalyst had to be made at intervals. Finally some 200 cc. of hydrogen were absorbed (calculated uptake 1220 cc.). From the reaction mixture 9·8 g. of a clear yellow oil were separated. On standing it deposited a crystalline material which was removed and recrystallised from methyl alcohol. Several fractions of this product, amounting in all to 0·17 g., were finally obtained in the form of white glistening plates. It melted at 67–69°, and appeared to be an incompletely purified hydrocarbon:

0·0176 g. gave 0·0544 g. CO_2 and 0·0330 g. H_2O.
Found: C, 84·28 %; H, 14·53 %.

The mother liquors from the crystalline hydrocarbon gave 8·9 g. of a clear red oil (II) with an iodine value of 118 and giving a fairly strong colour reaction for vitamin A. As no further crystalline material separated from this fraction after treatment with solvents it was distilled at 0·04 mm. Two fractions were obtained: (*a*) 2·05 g. boiling between 189–220°, I.V. 96, (*b*) 5·20 g. boiling between 220–270°, I.V. 138 Attempts were made to reduce these fractions more completely, as it was obvious that the original material had been very incompletely reduced, but they were unsuccessful, and only yielded small amounts of a product apparently identical with the impure hydrocarbon described above.

Our experience in carrying out the hydrogenation of the active fractions of cod-liver oil appears to have been in some respects similar to that of Nakamiya and Kawakami [1927]. The Japanese investigators found difficulty in carrying out a complete reduction when using palladium catalyst and temperatures up to 60°. From their treated products they isolated only small amounts of crystalline substances, accounting in all for some 5–10 % of the original "biosterin." The identification of some of their crystalline products is wholly unsatisfactory, but they claim to have detected nonocosane, batyl alcohol, melissyl alcohol and octadecyl palmitate. It is difficult to understand the presence of the latter substance in a material that is supposed to have

been completely saponified. The liquid fraction of their hydrogenated material was also a light brown oil, which did not solidify even at $-75°$.

Further attempts to carry out a more satisfactory separation of the vitamin fraction by careful distillations were made. The great loss of vitamin A which we observed seemed to be in part caused by the incomplete removal of cholesterol and other sterols precipitated by digitonin. In the earlier studies [Drummond, Channon and Coward, 1925] material treated by digitonin was used, and the loss of vitamin was certainly much less, although we have no quantitative measure of the proportions. We could not afford to use digitonin on the scale necessary to treat large amounts of the red oil (I) from the unsaponifiable matter of cod-liver oil, and all efforts to distil it without such treatment were unsuccessful in that however carefully the distillation was conducted there was always a serious loss of the active substance and, we believe, considerable general decomposition. The failure of these efforts, which occupied many months, to separate the constituents of the unsaponifiable matter from cod-liver oil by distillation, or to isolate appreciable amounts of crystalline substances after reduction, led us to turn our attention to other rich sources of vitamin A.

Before describing this extension of the work, attention may be directed to a minor point of interest which arose during the cod-liver oil studies.

Presence of squalene in cod-liver oil.

In the earlier study by Drummond, Channon and Coward [1925] the presence in cod-liver oil of a hydrocarbon closely resembling squalene was described. On the basis of the yield of the crystalline hexahydrochloride and the octodecabromide it was estimated that the unsaponifiable fraction of the cod-liver oil used in that investigation—a Newfoundland medicinal oil—contained approximately 8 % of squalene.

During a study of the distribution of squalene in a large number of marine species, Channon [1928] failed to detect this hydrocarbon in the material extracted from authentic cod-livers or the livers of other *Teleostei*. He suggested that the presence of squalene in commercial cod-liver oils might be due to admixture with liver oils from fish other than gadoids. A similar view had been advanced by Nakamiya and Kawakami [1927] who failed to detect squalene in their "biosterin" fractions. During the past year we have been able to examine a number of cod-liver oils of unquestioned purity from various sources, and have in every case detected the presence of small amounts of squalene, or a similar substance, by the formation of the characteristic hexahydrochloride. In all cases the test was carried out by carefully preparing the unsaponifiable fraction and removing the greater part of the cholesterol by freezing out from dry methyl alcohol. After removal of the solvent the residue was dissolved in dry acetone and saturated with dry hydrochloric acid. If a crystalline precipitate was formed it was filtered off and washed well with dry ether to remove cholesterol hydrochloride. The

insoluble portion was recrystallised from dry acetone. The following results were obtained:

Oil			Unsaponifiable %	Hydrochloride mg. per 100 g. oil	Hydrochloride M.P.
Norwegian.	Spring	1928	0·66	12	123°
Aberdeen.	,,	1928	0·68	2·9	126°
Moray Firth.	,,	1928	0·65	8·0	126°
Newfoundland.	Summer	1927	0·67	3·5	126°
,,	,,	1928	0·67	3·2	125°
North Sea.	,,	1928	0·80	8·3	122°

These figures seem to establish that small amounts of squalene or a very similar hydrocarbon do occur in pure cod-liver oil.

The high iodine values of fractions 4 and 5 obtained in the distillation of the red oil (I) described above appeared to be due to the presence of this hydrocarbon, as comparatively large amounts of the characteristic hexahydrochlorides were isolated. From 1 g. of fraction 4 there was obtained a yield of 156 mg. of the hydrochloride, whereas the same amount of fraction 5 gave 218 mg. This product on recrystallisation showed a melting point of 126° and contained 33·7 % of chlorine (calculated for squalene hexahydrochloride 33·86 %).

B. *Sheep-liver fat.*

The unsaponifiable matter was prepared from a concentrate placed at our disposal by Mr F. H. Carr, by one treatment with alcoholic KOH and one with sodium ethoxide. It was a dark reddish brown, rather hard wax with a vitamin value of 20 and containing 35 % of cholesterol. The greater part of this constituent was removed by crystallisation from methyl alcohol, and a residual dark red oil (III) was obtained with an iodine value of 171 and a vitamin value of 35. A portion of this oil was distilled at a pressure of 0·012 mm. The distillation was very satisfactory as regards temperature control, and there were no apparent signs of decomposition. Nevertheless, a steep distillation curve (Curve *B*, Fig. 1) was obtained indicating that the mixture was either a very complex one or that decomposition had occurred.

The following fractions were, however, collected:

Fraction No.	Wt. g.	Appearance	Temp.	Iodine value	n^{40}	Vitamin value
1	2·7	Clear brown-yellow liquid	177°–190°	113	1·4861	6·7
2	2·0	Opaque brown liquid	190°–220°	149	1·4876	15·3
3	0·5	Semi-solid yellow mass	220°–230°	171	1·5043	8·3
4	2·8	Hard yellow wax	230°–260°	125	1·5089	3·7
5	2·7	Brownish yellow wax	260°–280°	138	1·5178	0·5

Of the original 16 g. of oil distilled only 10·7 g. were recovered in the fractions, and the residue in the flask was a thick black tar smelling strongly as if decomposition had occurred. Evidence of decomposition was also given by the very strong terpene-like smell of the distilled fractions.

Although some of the fractions showed fairly strong colour reactions it was apparent that the loss of vitamin A had been considerable from a calculation of the recovery (11 %) of chromogenic substance. Fractions 4 and 5 were combined and recrystallised from methyl alcohol. Nearly 1 g. of cholesterol was separated. The mother-liquors gave a deep red coloured oil (IV) from which no further crystalline material was obtained on treatment with various solvents. Fractions 1 and 2 were also combined and a small amount of cholesterol removed by precipitation with digitonin. The residual red oil (V) had an iodine value of 125, and was treated with hydrogen in the presence of palladium catalyst. It proved as resistant to reduction as the similar fractions from cod-liver oil, and after a long period in contact with the gas, and after several renewals of the catalyst, the iodine value had only fallen to 55.

It seemed possible that some of the difficulties encountered in the examination of this material might be due to the presence of the curious hydrocarbon found by Channon and Marrian [1926] in the liver fats of certain mammals. In order to test this possibility a small quantity of the fraction was tested by saturating the dry acetone solution with hydrochloric acid gas. A crop of a crystalline material was obtained, which differed in crystalline form from squalene hydrochloride and appeared to resemble the hydrochloride described by Channon and Marrian. A further 6 g. of the original unsaponifiable material (III) were freed completely from cholesterol, and fractionated from methyl alcohol in the manner described by these investigators. The following fractions were separated:

		I.V.
(*a*)	1·27 g.	123
(*b*)	0·3	134
(*c*)	0·11	154
(*d*)	0·30 (insoluble residue)	302

The iodine value of the corresponding fraction insoluble in methyl alcohol separated from pig-liver material by Channon and Marrian was 309. Bromination of our insoluble material gave 0·68 g. of the bromide similar in character to that described by them. This yield and an iodine value of 302 would correspond to the fraction containing about 81 % of the hydrocarbon with an iodine value of 370. A bromination of 0·5 g. of the original sterol-free unsaponifiable matter gave 0·275 g. of insoluble bromide. This corresponds with the material containing approximately 18 % of the hydrocarbon, and would allow an iodine value of about 80 for the other constituents.

The importance of the detection of such relatively large amounts of this curious hydrocarbon in the unsaponifiable fraction of sheep-liver fat lies in the observation of Channon and Marrian that it is readily decomposed when distilled under reduced pressure. A considerable production of liquids of low boiling point, together with a failure to obtain any bromide or hydrochloride from the distillates, seemed to indicate that the decomposition was extensive.

It is, we believe, decomposition of this type that is responsible for the loss of vitamin A by secondary reactions when the unsaponifiable material of cod-liver or sheep-liver oil is distilled under conditions that might be regarded as unlikely to cause destruction of the active substance.

An attempt was made to reduce with hydrogen and palladium the material more soluble in methyl alcohol obtained during the separation of the hydrocarbon. It was thought that the removal of the greater part of the hydrocarbon, itself of a constitution not readily reduced, might render the reduction of the residue more readily accomplished. Such did not prove to be the case. A solution of 1·79 g. dissolved in glacial acetic acid was treated with hydrogen under a pressure of about an atmosphere and a half. Hydrogen was very slowly absorbed, and several renewals of catalyst were made. In 5 hours 170 cc. of gas were absorbed (calculated 190 cc.), and the reduction seemed to have ceased. After hydrolysis of the reduced product, in case acetylation had occurred, 1·6 g. of a viscous golden yellow oil were obtained. This material failed to yield any crystalline product.

C. *Greenland shark-liver oil.*

The studies of Tsujimoto and Toyama [1922] and Weidemann [1926] suggested that the liver oil of the Greenland shark, *Somniosus microcephalus*, might yield an unsaponifiable fraction of simpler composition than the materials we have already described, and therefore more likely to provide information regarding the nature of the vitamin. The unsaponifiable fraction of this oil is known to consist to a large extent of cholesterol and the two dihydric alcohols, batyl alcohol and selachyl alcohol.

The sample of Greenland shark-liver oil employed in our work was a commercial preparation of dark orange colour, containing 15 % of unsaponifiable matter. From it 566 g. of this material were prepared. The iodine value of this fraction was 57, it contained 14·5 % of cholesterol and it possessed a colour index of 1·5. The relatively low vitamin value of this material did not deter us from examining its composition in view of the results we were obtaining at the same time in the examination of the vitamin-rich Japanese shark-liver oil (see Section D).

On attempting to separate the greater part of the cholesterol by washing with ice-cold methyl alcohol a large amount of insoluble pale yellow crystalline material separated. This was further washed with repeated changes of methyl alcohol at 0° and gave nearly one-third of the whole unsaponifiable fraction in the form of a white crystalline product. These crystals were obviously a mixture as they melted indefinitely between 65 and 100°. It appeared probable that in addition to the greater part of the cholesterol, the methyl alcohol had separated a solid alcohol of the type of batyl alcohol.

By repeated fractionation of a representative sample from light petroleum about half the expected amount of cholesterol was isolated in reasonably

pure condition, but as regards the other components only a number of fractions with melting points ranging from 60° to 90° were isolated. A few of these fractions were combined, and the cholesterol was removed by digitonin. The residue was a white solid, which, after several recrystallisations from methyl alcohol, melted at 68–69°. This appeared to be identical with batyl alcohol, M.P. 70–71°, and a mixed melting point determination with a sample of batyl alcohol, for which we are indebted to Prof. Tsujimoto, showed no depression.

A better method for the separation of the batyl alcohol and cholesterol, which together appear to make up practically the whole of that part of the unsaponifiable material insoluble in methyl alcohol, is to employ ether as a solvent. Batyl alcohol is sparingly soluble in cold ether and considerable amounts were separated in this manner. The purified product melted sharply at 69°:

Analysis. 0·1205 g. gave 0·3240 g. CO_2 and 0·1370 g. H_2O.
Found: C, 73·20; H, 12·62.
Calculated for $C_{21}H_{44}O_3$: C, 73·25; H, 12·79.

The fraction of the unsaponifiable matter soluble in methyl alcohol weighed 306 g., and was an orange coloured oil with a vitamin A colour value of 1·3.

Several very satisfactory distillations of this product were made. One of them is represented by curve C, Fig. 1, from which it may be seen that the chief part of the distillate is probably represented by one substance. The data regarding the fractions taken in this particular distillation of 45 g. are as follows:

Fraction No.	Wt. g.	Appearance	Temp.	Pressure mm.	Iodine value	Cholesterol %	$n^{40°}$
1	5	Yellow semi-solid mass	150°–235°	0·015	68·2	0·74	1·4578
2	7·9	Cream coloured semi-solid fat	240°	0·042	75·3	2·82	1·4631
3	5·4	Pale yellow liquid	240°–244°	0·045	81·4	3·4	1·4640
4	2·8	Clear brownish liquid	244°–250°	0·077	89·6	4·54	1·4623
5	9·7	,,	250°–254°	0·10	91·2	3·98	1·4649

Of the 45 g. taken 26 g. boiled between 240–254° and appeared to be uniform, apart from a slight rise in iodine value as the distillation proceeded. The properties of these fractions suggested that they might be largely composed of unsaturated substances of the type of selachyl or oleyl alcohol.

	I.V.	n^{40}
Selachyl alcohol	79	1·4691
Oleyl alcohol	94	1·4620

Very small amounts of a hydrocarbon, probably squalene, were detected by the formation of a crystalline hydrochloride.

2 g. of the undistilled fraction gave 18·7 mg. hydrochloride, equivalent, assuming a 50 % yield, to the presence of 0·6 % of squalene.

Only the first three fractions showed the colour reaction for vitamin A.

These were too pale to measure. 16 g. of the material, boiling between 240–254°, fractions 3, 4 and 5, were redistilled.

Fraction No.	Wt. g.	Temp.	Pressure mm.	Iodine value	$n^{40°}$
1	1·9	120°–130°	0·01	104·1	1·4561
2	4·2	150°–170°	,,	87·6	1·4552
3	1·6	170°–180°	,,	87·6	1·4570
4	2·8	180°–195°	,,	84·6	1·4619
5	1·7	195°–203°	,,	87·9	1·4722

Fractions 2–5 appeared to be uniform and were combined (fraction 134). 4·3 g. of the lower boiling material (fractions 1 and 2) from the main distillation were also refractionated. They yielded 2·7 g. of a semi-solid white wax boiling at 180–200° at 0·01 mm., with an iodine value of 74, and $n^{40°}$ 1·4630 (fraction 132).

Fraction 132. Although this material was semi-solid no satisfactory crystallisation from solvents was carried out, and it was subjected to hydrogenation. 2 g. dissolved in 100 cc. alcohol were treated with hydrogen and palladium catalyst. Absorption of hydrogen was rapid and a white crystalline material separated out in the reacting vessel during the later stages of the treatment.

The reduced substance (1·9 g.) was a white crystalline material melting at 53·54°. After recrystallisation from acetone, fractions of a white substance crystallising in clusters of very small, fine, curved needles were obtained, M.P. 60–61°. This material was later combined with similar fractions obtained by the reduction of fraction 134.

Fraction 134. Cholesterol was removed quantitatively from this material by digitonin leaving a brown oil; I.V. 86.

It was reduced with hydrogen and palladium very readily, and yielded a white crystalline product melting at 45–50°. After two recrystallisations from acetone it melted at 65–66°.

Fractionation of crystalline material from hardened fractions 132 *and* 134.

As it appeared likely that the products of hydrogenation of fractions 132 and 134 were very similar in composition they were combined for a systematic fractionation from acetone. The less soluble fractions all with melting points above 59° were united and recrystallised from methyl alcohol. The melting point of the product was 64–65°.

Analysis. 0·0722 g. gave 0·1942 g. CO_2 and 0·0812 g. H_2O.
Found: C, 73·35 %; H, 12·63 %.
Calculated for $C_{21}H_{44}O_3$: C, 73·25 %; H, 12·79 %.

The phenylurethane melted at 96–97·5°. This product appeared, therefore, to be batyl alcohol. Heilbron and Owens [1928] give the melting point of the urethane as 98°. The fractions more soluble in acetone, with

melting points below 59°, were combined and 2·75 g. were redistilled at 0·06 mm.

Fraction No.	Wt. g.	Temp.
1	0·5	140°–155°
2	0·65	155°–191°
3	0·55	190°–200°
4 Residue	0·7	—

Each fraction was recrystallised from methyl alcohol. The melting points of the recrystallised materials were (1) 51·8°, (2) 54·5°, (3) 56·6°, (4) 57·5°.

Fractions 1 and 2 were analysed:

(1) 0·0768 g. gave 0·2190 g. CO_2 and 0·0930 g. H_2O.

Found: C, 77·76 %; H, 13·45 %.

(2) 0·0762 g. gave 0·2157 g. CO_2 and 0·0904 g. H_2O.

Found: C, 77·20 %; H, 13·18 %.

Calculated for octadecyl alcohol ($C_{18}H_{38}O$): C, 79·91; H, 14·17.
Calculated for batyl alcohol ($C_{21}H_{44}O_3$): C, 73·25; H, 12·79.

The phenylurethanes were prepared, and melted at 71–73°. Heilbron and Owens [1928] give the melting point of the phenylurethane of octadecyl alcohol as 77–78°; André and Francis [1926] give 79–80°. Attempts were made to purify the parent substances, but it was found to be a very difficult matter. The material isolated from these hardened fractions behaved very much like a mixture of batyl and octadecyl alcohols, when compared with mixtures of known composition of the two substances. Prof. Heilbron informs us that he has encountered the same difficulty in separating mixtures of the urethanes of these two substances.

From these studies we concluded that the unsaponifiable matter from the liver oil of the Greenland shark, consists to a large extent of cholesterol, batyl alcohol and unsaturated alcohols—probably selachyl and oleyl—which on reduction yield respectively batyl and octadecyl alcohols.

D. *Japanese shark-liver oil.*

Through the courtesy of Mr John Spencer of Aberdeen we were able to obtain a sample of commercial Japanese shark-liver oil of relatively high vitamin value. This oil contained approximately 5·5 % of unsaponifiable matter and gave a vitamin test of 0·2. The unsaponifiable fraction was a clear pale yellow oily fluid, I.V. 78, and on standing a slight deposit of crystalline material separated. Small amounts of cholesterol were found. 160 g. of this unsaponifiable material were recrystallised from methyl alcohol and yielded 34 g. of crystalline substance. The melting point of this product, after many recrystallisations from light petroleum and ether, rose to 62–62·5°. It crystallised in sheaves of needles from ether, in which it is not very soluble. It appeared to be identical with chimyl alcohol, $C_{19}H_{40}O_3$, described by Toyama [1924], M.P. 60·5–61·5°.

Analysis. (1) 0·0707 g. gave 0·1880 g. CO_2 and 0·0821 g. H_2O.
Found: C, 72·51 %; H, 12·9 %.
(2) 0·0653 g. gave 0·1725 g. CO_2 and 0·0747 g. H_2O.
Found: C, 72·03 %; H, 12·71 %.
Calculated for $C_{19}H_{40}O_3$: C, 72·14 %; H, 12·66 %.

The phenylurethane crystallised in long needles from ethyl alcohol, M.P. 98°.

Treatment of chimyl alcohol with hydriodic acid. In order to isolate and identify the fatty alcohol present in the chimyl alcohol we reduced it with hydriodic acid in a micro-Zeisel apparatus so that we were enabled to make an estimation of the glycerol as *iso*propyl iodide at the same time. The reduction was carried out in the following manner. The reaction flask was heated slowly to 110° and maintained at that temperature for half an hour. The temperature was then gradually raised to 130° for a further 30 mins., and finally to 145–150° for 10 mins. Under these conditions we obtained yields of silver iodide corresponding to 23·19, 23·85 and 23·15 % glycerol. The theoretical value for chimyl alcohol is 29·12 %. We have not succeeded in obtaining values nearer the theoretical than those given above, although we have attempted to modify the conditions of the reaction so as to render smaller the likelihood of secondary reactions occurring. Parallel determinations on pure samples of batyl alcohol gave values of 21·5, 21·97 and 22·02 % as compared with the calculated figure of 26·74 %.

After the treatment with hydriodic acid the contents of the flask in the case of the experiments with chimyl alcohol contained an oily layer, whereas in the case of batyl alcohol the layer solidified to a soft wax. The oily layer in the former case was extracted with ether, and washed with sodium bisulphite and with sodium bicarbonate solutions. The crude iodide was recrystallised twice from acetone and melted at 23–25°. This product appeared to be cetyl iodide, M.P. 23° [Gascard, 1921]. Some of the iodide was converted into the corresponding alcohol by treatment with sodium acetate and subsequent saponification. The cetyl alcohol after recrystallisation from acetone melted at 44–47°.

These observations serve to confirm the constitution of a monoglyceryl ether of cetyl alcohol for chimyl alcohol suggested by Heilbron and Owens [1928] as a result of their studies, which revealed batyl alcohol to be an ether of octadecyl alcohol and glycerol.

The fraction of the unsaponifiable matter soluble in methyl alcohol deposited on standing a considerable amount of additional crystalline material which was separated by centrifuging. Both solid and liquid fractions were treated with digitonin to remove a small amount of cholesterol. The liquid oil after this treatment possessed an iodine value of 103 and a vitamin value of 3·75.

35 g. of this oil were distilled at 0·04 mm., and the following fractions collected:

Fraction No.	Wt. g.	Appearance	Temp.	Iodine value	n_D^{20}	Vitamin value
1	1·7	Yellow-brown liquid, some crystals	120°–190°	69	1·4720	3·3
2	2·6	,,	190°–202°	84	1·4762	4·0
3	8·2	Yellow semi-solid mass	202°–205°	100	1·4755	3·3
4	7·9	,,	205°–206°	101	1·4731	1·4
5	8·1	,,	206°–208°	101	1·4725	0·6
6	4·2	Yellow wax	208°–210°	103	1·4725	0·4
7	3·3	Hard yellow wax	210°–220°	107	1·4769	1·0

As shown by the distillation curve *D* (Fig. 1) this material seems to be uniform, and the results of the examination of the fractions confirm this view. The recovery of vitamin was nearly 50 % and in fraction 2 there was actually a slight concentration of the active substance. As regards the vitamin this was the most successful distillation we have had in the course of 5 years' work. Fractions 3 and 4 were reduced with hydrogen in the presence of palladium at room temperature, the absorption of hydrogen being very rapid. From both fractions a white crystalline material was isolated in quantitative yield. Even in the crude condition it appeared to be fairly pure batyl alcohol, and on recrystallisation from ether this substance was obtained readily, M.P. 67–69°; phenylurethane, M.P. 98·5–99°. The small amounts of material in the mother-liquors from the purification of the batyl alcohol were soft waxes with iodine values of about 74. They contained batyl alcohol, but there was insufficient to isolate the other constituents. They did not give a colour reaction for vitamin A. Small traces of the hydrocarbon like squalene were detected in the distilled fractions, but it is doubtful whether they were sufficient to account for the iodine values of 102–107 on the assumption that the main constituent was selachyl alcohol (I.V. 79).

The solid material that deposited on standing from the methyl alcohol-soluble fraction of the unsaponifiable matter was also distilled at 0·01 mm. after removal of the cholesterol by digitonin. It had an iodine value of 90 and a vitamin value of 3·1. The following fractions were collected:

Fraction No.	Wt. g.	Appearance	Temp.	Iodine value	n_D^{20}	Vitamin value
1	0·9	Yellow-brown semi-solid wax	90°–180°	61	—	1·2
2	4·4	,,	180°–195°	81	1·4790	1·9
3	9·4	,,	195°–204°	87	1·4788	0·7
4	10·8	Pale yellow wax	204°–208°	90	1·4778	0·3
5	2·2	Brown wax	208°–227°	100	1·4755	0·6

The distillation curve is shown in curve *E*, Fig. 1, and is very similar to that of the corresponding liquid fraction (curve *D*). The recovery of vitamin is not so good, 23 % as compared with 48 %, but in this case there was some preliminary manipulation which may slightly have damaged the product. The information regarding the fractions again suggests that mainly one substance is present, and confirmation of this was obtained from the reduction

experiments which yielded almost quantitative amounts of batyl alcohol, M.P. 68–69°; phenylurethane, M.P. 98·5°.

It would seem, therefore, that the greater part of the fraction of the unsaponifiable matter soluble in methyl alcohol is selachyl alcohol. (Selachyl alcohol, B.P. (5 mm.) 236–239°, I.V. 79, $n^{20°}$ 1·4691.) An analysis was made of fraction 4:

(a) 0·0832 g. gave 0·2262 g. CO_2 and 0·0925 g. H_2O.
Found: C, 74·14 %; H, 12·28 %.

(b) 0·0806 g. gave 0·2192 g. CO_2 and 0·0893 g. H_2O.
Found: C, 74·16 %; H, 12·31 %.

Calculated for $C_{21}H_{42}O_3$: C, 73·69 %; H, 12·28 %.

Determinations of glycerol on the crude hydrogenated fractions (M.P. 58–60°) gave 22·24 and 22·11 %, figures agreeing very well with those obtained on pure batyl alcohol.

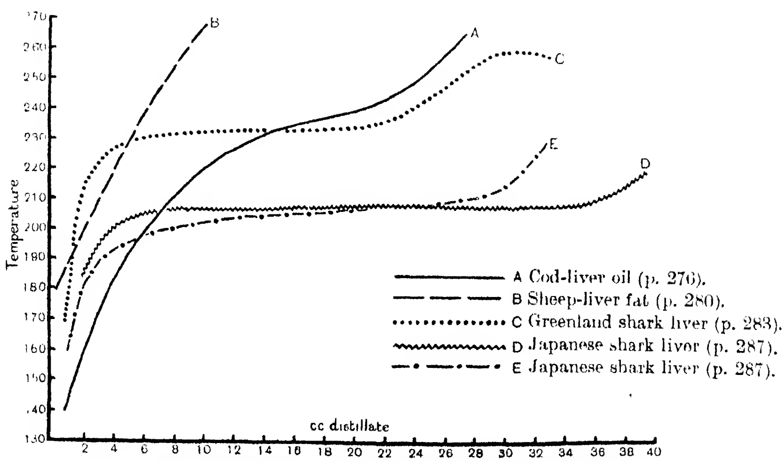

Fig. 1. Distillation curves of unsaponifiable fractions.

Only the fact that most of the fractions were more or less solid waxes was against their being almost wholly composed of selachyl alcohol. Pure selachyl alcohol freezes at about 5–10°, but it remains a liquid at temperatures at which our fractions were solid. It is possible that the substance present in the material we were examining was an isomer of selachyl alcohol. We are investigating this matter further.

Discussion.

Viewed from the standpoint of the main object of the investigation, namely, to ascertain the chemical nature of vitamin A, the observations recorded in this paper are unimportant. They serve, however, to make it clear that the task of isolating this vitamin is one of even greater difficulty

than our previous experience had led us to believe. The results obtained in the study of the Japanese shark-liver oil are sufficient to show that the vitamin forms a very minute proportion of the total unsaponifiable matter. The original oil possessed a vitamin A value comparable with that of a very good cod-liver oil. From the unsaponifiable fraction were isolated, either directly or after reduction with hydrogen, recognisable substances in reasonably pure condition in amounts accounting for 90 95 % of the total material. As far as could be ascertained the residue consisted to a large extent of these same substances in less pure condition.

It is generally recognised that the separation of the components of mixtures of fatty substances of low melting points is in all cases exceedingly difficult, and that it is well-nigh impossible if the desired substance represents a small proportion of the mixture. After removal of the cholesterol and chimyl alcohol from the unsaponifiable fraction of the Japanese shark-liver oil a residue was obtained which could be regarded as slightly impure selachyl alcohol.

It distilled completely over a narrow range of temperature, and on treatment with hydrogen yielded almost quantitatively the corresponding saturated compound, batyl alcohol. We are forced, therefore, to conclude that the vitamin is present in amounts so small that its separation will not be attained by such methods as we have used, unless it be found that a characteristic derivative can be prepared which possesses properties suitable for its isolation.

In illustration of this view there can be given the case of the hydrocarbon (squalene) that was found in some of the fractions. Fractions 4–6 of the distillation described on p. 287 appeared to consist almost entirely of selachyl alcohol, but possessed an iodine value (100–103) which suggested that a small amount of an impurity with a much higher degree of unsaturation than that substance was also present. Owing to the fortunate fact that very small amounts of this hydrocarbon can be detected by the formation of the insoluble hydrochloride or bromide it was possible to demonstrate that some, if not all, of the excess of the iodine value of these fractions over that of selachyl alcohol could be accounted for in this manner. Only 7 % of squalene would be required to raise the iodine number of these fractions from 79 (selachyl alcohol) to the observed value of 100, and yet, were the separation of the bromide and hydrochloride not an easy matter, the detection, much more the isolation, of this constituent would be an exceedingly difficult task.

The results reported in this paper throw no definite light on the chemical nature of vitamin A, nor do they provide reasonable grounds for speculation. So far only one indication has been obtained, and it is one that we think points the direction in which work might profitably be directed in the future. The colour reactions believed by many to be specific for vitamin A recall so strongly those given by certain types of sterol derivatives, that it is probable that more progress in elucidating the nature of the active substance will be made by studying the properties of sterols than by continuing to employ such

methods as we have used during the last ten years. It is encouraging to recall the brilliant success which has followed the studies of the sterols in relation to vitamin D, and to remember that the concentrated preparations of the antirachitic vitamin that are now available would probably never have been obtained by employing such methods as we have used in attempting the isolation of vitamin A.

Summary.

1. Further attempts have been made to separate by fractional distillation at pressures of about 0·01–2 mm. the vitamin A present in the unsaponifiable matter of certain liver oils. These efforts have not succeeded.

2. The unsaponifiable fraction of cod-liver oil, after removal of the greater part of the cholesterol, does not fractionate satisfactorily, but tends to decompose with rather serious loss of the vitamin.

3. The constituents of the unsaponifiable fraction of cod-liver oil are not readily reduced by hydrogen in the presence of platinum or palladium catalysts, and little information regarding their nature was obtained by this line of attack. It was also found impracticable to effect their separation by the preparation of phthalates or substituted phthalates.

4. The unsaponifiable fraction from sheep-liver fat also decomposed considerably on distillation in a high vacuum. Part of this decomposition, which involves the vitamin, is due to the presence of the highly unsaturated hydrocarbon, resembling in some respects squalene, discovered by Channon and Marrian in mammalian livers. Considerable amounts of this substance were separated from the vitamin fractions.

5. The sheep-liver fractions were as resistant to hydrogenation as those from cod-liver oil.

6. The unsaponifiable fractions of Greenland shark-liver oil and Japanese shark-liver oil consist largely, as the studies of Tsujimoto and Toyama have indicated, of selachyl, batyl, chimyl and oleyl alcohols.

7. The distillation of the unsaponifiable fractions from these oils is accompanied by comparatively little destruction of the vitamin, owing, it is thought, to the small proportion, or absence, of the complex alcohols and hydrocarbons of the terpene series.

8. A sample of Japanese shark-liver oil possessing a vitamin activity of the same order as a good cod-liver oil yielded an unsaponifiable fraction of which 90–95 % was accounted for in the form of reasonably pure preparations of the alcohols mentioned in 6, together with small amounts of cholesterol and a hydrocarbon resembling squalene. The remainder appeared to consist to a large extent of the same substances. This would indicate that the vitamin A forms a very small proportion of the unsaponifiable matter, probably less than 1 %, and supports the view expressed formerly by us that the "biosterin" of Takahashi and his colleagues is an extremely crude preparation of the active substance.

9. The structure suggested by Heilbron and Owens [1928] for chimyl alcohol, namely, that of a monoglyceryl ether of cetyl alcohol, has been confirmed by using methods similar to those employed by them in determining the constitution of the related batyl alcohol.

We are greatly indebted to the Medical Research Council for a series of grants during the past few years that have defrayed the cost of this investigation.

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XXXVII. MOLECULAR CONSTITUTION AND ACCESSIBILITY TO ENZYMES.

THE EFFECT OF VARIOUS SUBSTANCES ON THE VELOCITY OF HYDROLYSIS BY PANCREATIC LIPASE.

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Introduction.

It has been shown by Quastel and Wooldridge [1928] that in the case of the dehydrogenating enzymes of the bacterial cell, the question of accessibility to the active centres is closely bound up with the molecular constitution of the substance under consideration.

It was suggested by Dr Quastel that it would be instructive to investigate a so-called soluble enzyme, *i.e.* one presumably not capable of being considered part of a cellular interface, and to find whether any substances possessing a particular molecular structure, and other than the natural substrates, are adsorbed at its surface, using the same general criterion, viz. that, if the substance is adsorbed, it will compete with the substrate for the available surface and diminish the velocity of the normal reaction.

It followed from the active centre hypothesis that a number of substances characterised by the possession of a particular type of structure should be adsorbed by (or capable of combining with) each enzyme or centre; but out of these substances only a certain number can be activated to undergo the chemical reaction. Substances which are adsorbed but not activated will compete for the active centre with those which are activated and hence bring about a diminution in the velocity of reaction of the latter. The facts found with the dehydrogenating enzymes supported these conclusions, but it was desirable to determine whether a study of a hydrolytic enzyme would also give confirmatory results. The investigation to be described therefore was primarily taken up to examine the active centre hypothesis from a study of hydrolytic enzymes.

The lipases were selected for study as they are not specific to anything like the same degree as the carbohydrases, and it should therefore be expected that a larger number of compounds would be accessible. Nevertheless in this connection Armstrong [1904] showed that for the sugar-splitting enzymes substances could be found which, though not attacked by the enzyme, inhibited its action on substrates, thus α-methylgalactoside, which is unattacked by lactase, inhibits the hydrolysis of lactose by that enzyme. The peptidases

also show specificity only of a limited degree, but lipases possess the advantage that the substrates are much more readily obtained in a state of purity.

Method and apparatus.

The aim of the experiments being to observe the effects of added substances on the velocity of hydrolysis, and not in any way on the total amount hydrolysed either up to an equilibrium or in any given time, all such methods of measurement as incubation for a standard time were unsuitable, and it was necessary to find a method by which the actual velocity at any moment could be readily calculated.

For this purpose the gasometric method was considered the most suitable. The principle of this method, in which the extent and velocity of liberation of acid in a system is measured by observing the output of carbon dioxide from a carbonic acid-bicarbonate buffer, is due to Warburg [1923], who employed it in the investigation of lactic acid formation in tissues. It was first applied to the study of lipases by Rona and Lasnitzki [1924], who used a micro-method carried out in ordinary Barcroft manometers. Both cups contained Ringer's solution containing bicarbonate, and the air was displaced by a gas mixture containing a fixed percentage of carbon dioxide. Enzyme, in their case diluted serum or tissue slices, was added to each cup and substrate to only one. By this means the time-course of the hydrolysis could be accurately followed.

For the present work the apparatus used by Dann and Quastel [1928] for the study of the rate of carbon dioxide production in fermentation was employed. By means of this apparatus, velocities of carbon dioxide output up to 1 cc. in 30 or 40 seconds can be measured with reasonable accuracy.

The chief advantage of this method, in addition to the fact that the actual velocity can be followed, is that the hydrogen ion concentration is automatically kept constant, as free acid is never able to accumulate in the system under the experimental conditions. The hydrogen ion concentration of a system carbonic acid-bicarbonate is given by $[H]^+ = K \frac{[H_2CO_3]}{[HCO_3^-]}$ or approximately by $K \frac{\text{free } CO_2}{\text{combined } CO_2}$ or $K \frac{\text{acid}}{\text{salt}}$, where K is the first dissociation constant of carbonic acid. If the concentration of the salt initially is sufficiently great, the amount decomposed makes a negligible difference, and the numerator term remains the same also to a first approximation so long as the partial pressure of the carbon dioxide remains constant. Since an actual halving of the concentration of salt, leading to a doubling of the actual hydrogen ion concentration, would only increase the p_H by 0·3, which would not materially affect the activity of the enzyme over the range employed, it will be seen that the error from this source is small. The largest error is found to be that due to irregularity in the speed of the stirring mechanism. The speed of the stirrer must be maintained at a value high enough to make the water surface a steep funnel, otherwise the liquid tends

to become supersaturated with carbon dioxide. In such circumstances not only is the rate of evolution of gas not constant, but also the mean rate is not comparable with the normal, since the ionic equilibrium controlling the p_H is disturbed.

The actual value of the p_H could be deduced from the equation, but is more conveniently found by experimental determination. To obtain any desired value for this quantity either the numerator or the denominator may be varied. Under the technique of Rona and Lasnitzki referred to, the p_H was adjusted by varying the percentage of CO_2 in the gas mixture filling the apparatus. The amount of extra CO_2 liberated in the experiment was of the order of 5 to 10 micro-mols only and did not affect the concentration. In the method here described, however, large volumes of liberated CO_2 are dealt with, and successive amounts of 5 cc. are, after measurement, expelled from the apparatus. A gas mixture is therefore impossible, and the whole air in the apparatus must always be displaced by CO_2 before the start of the experiment. For this reason the bicarbonate concentration had also to be much greater in order to obtain a p_H of the order of 7, in fact the concentration required was about $M/7$. This concentration however was found to have no deleterious effect on the enzyme.

Preparation of the enzyme.

Nearly all the experiments were carried out with pancreatic lipase of the pig. A dry preparation of pig's pancreas, free from fat, was prepared by drying with acetone and ether after the procedure of Willstätter and Waldschmidt-Leitz [1923]. The dry preparation appears to keep indefinitely if stored in the ice-chest. Aqueous extracts were made as desired, employing 16 cc. distilled water for each gram and shaking intermittently for 3 to 4 hours at room temperature and centrifuging. Such extracts can be kept for a week without toluene if regularly replaced in the ice-chest after use; and under such treatment their activity, after falling during the first 12 hours, remains constant for about 5 days. Subsequently the activity rapidly falls off, but samples in this stage were always discarded.

Experimental procedure and types of result.

The contents of the reaction vessel, which held about 70 cc., were made up of 40 cc. $M/4$ sodium bicarbonate solution, 25 cc. distilled water and 5 cc. of the solution containing the enzyme. The bicarbonate solution was usually saturated with washed CO_2 from a cylinder, but in any case the whole mixed contents were saturated with the gas during the replacement of the air in the apparatus by CO_2, the gas passing through the liquid from the outlet tap at the bottom. The substrate, generally 1 cc., was added last, the taps being then closed to leave the capillary leading to the burettes the only outlet for gas, and the apparatus placed in the bath, the water in which had been

already adjusted to the correct temperature, and the stirrer started. The bath was maintained at a temperature of 30°.

The apparatus was left for 5 minutes or more to attain equilibrium, the approximate time being known from experiments in which the substrate was not added until pressure equilibrium was obtained. In any case readings were discarded before the point at which a constant velocity of gas evolution was first observed.

It was found that linear curves for the hydrolysis could be obtained over considerable periods with this technique. The actual extent was found to vary markedly from substrate to substrate. The most satisfactory results were obtained when using ethyl butyrate as substrate, the linear course being followed up to 40 or 50 % hydrolysis. The phenomenon is probably attributable to the properties of the products of hydrolysis, the alcohols more particularly than the fatty acid salts. Thus, while ethyl butyrate and other

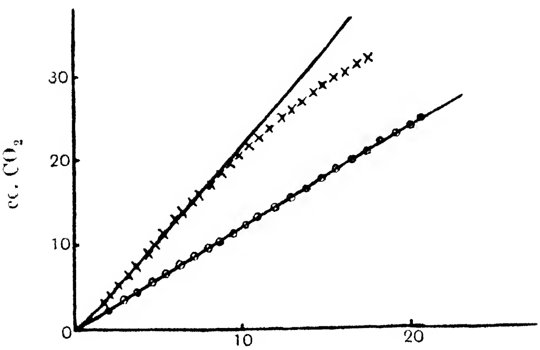

Fig. 1.

× × × × Triacetin. ⊙ ⊙ ⊙ ⊙ Ethyl butyrate.

ethyl esters maintain a linear course up to 50 % hydrolysis as stated, glycerides give a much shorter period and butyl and amyl esters fail to maintain a linear course at all. In correlation with this, butyl and amyl alcohols cause the greatest inhibition when added to the system, although the observed difference between glycerol and ethyl alcohol is not very noticeable. Fig. 1 shows the different course followed by the hydrolyses of ethyl butyrate and of triacetin. It will be observed that in the latter case the linear course does not persist longer than 10 minutes, while no deviation is to be noticed at all in the case of the ethyl butyrate.

Rona and Lasnitzki also state that the linear course of hydrolysis of tributyrin in their micro-technique persisted only up to the liberation of 2·5 micro-mols of butyric acid or about 7·5 % hydrolysis. That this is not due to the products of partial hydrolysis, dibutyrin and monobutyrin, having a greater affinity for the enzyme than the tributyrin itself, is easily shown. Although the velocity of hydrolysis of these compounds by pancreatic lipase

is much smaller than the velocity of hydrolysis of tributyrin, their addition to a system of tributyrin and enzyme does not cause a fall in the velocity. This was shown by Terroine [1910] and was confirmed by the writer for triacetin and monoacetin.

Hydrolysis of mixtures of substrates.

The above case is merely a general example of the study of mixtures of substrates. Where such mixtures were used in these experiments, it was found in nearly all cases that the velocity of hydrolysis was equal to the velocity of hydrolysis of the more rapidly hydrolysed constituent taken singly. Thus the addition of ethyl butyrate to the apparatus while triacetin was being hydrolysed led to no alteration in the velocity of gas evolution, but triacetin added to the system hydrolysing ethyl butyrate increased the velocity to that observed with triacetin alone. The following figures represent parts of a pair of such experiments:

A. Triacetin added to ethyl butyrate.

1 cc. CO_2 in	1′ 25″	1′ 26″	1′ 32″	*	56″	59″	57″	59″
2 cc. ,,	2′ 55″	3′ 0″	3′ 7″	*	1′ 54″	1′ 59″	1′ 55″	1′ 57″
3 cc. ,,	4′ 25″	4′ 37″	4′ 48″	*	2′ 53″	2′ 58″	2′ 49″	2′ 57″
4 cc. ,,	5′ 57″	6′ 17″	6′ 24″	*	3′ 46″	3′ 55″	3′ 46″	3′ 56″

B. Ethyl butyrate added to triacetin.

1 cc. CO_2 in	38″	36″	38″	*	39″	38″	39″
2 cc. ,,	1′ 15″	1′ 12″	1′ 17″	*	1′ 17″	1′ 16″	1′ 17″
3 cc. ,,	1′ 55″	1′ 49″	1′ 56″	*	1′ 54″	1′ 55″	1′ 54″
4 cc. ,,	2′ 32″	2′ 27″	2′ 35″	*	2′ 32″	2′ 34″	2′ 30″

* represents point of addition.

It is known however from the results of other workers that this phenomenon is not an unalterable rule. Thus Willstätter, Kühn, Lind and Memmen [1927], investigating the so-called latent period in the hydrolysis of ethyl mandelate by liver esterase, found that it was due to the presence of traces of ethyl phenylglyoxylate, the ester of the corresponding keto-acid, which they concluded was adsorbed much more strongly than the mandelate but hydrolysed more slowly. This compound is however an ester of a keto-acid, and the results to be described suggest that another phenomenon may be involved here.

It is now recognised that the velocity of hydrolysis of an ester by lipase is the result of two factors, the affinity of the enzyme for the substrate (represented by the Michaelis constant), and the velocity of decomposition of the enzyme-substrate complex. There is no reason to suppose that the relation between these should be the same for all substrates, and indeed it is not. Thus, of several pairs of optical isomers, that one which separately is less rapidly hydrolysed is found to be preferentially attacked in the optically inactive form. This phenomenon has been particularly studied by Willstätter, Kühn and Bamann [1928] and by Rona and Itolsohn-Schechter [1928].

The point to be stressed is that in no case is the velocity of hydrolysis of a mixture of substrates greater than that of either separately, that is to say,

there is no evidence that there is more than one enzyme concerned in the hydrolysis of all substrates.

EFFECT ON THE VELOCITY OF HYDROLYSIS OF COMPOUNDS CHEMICALLY UNRELATED TO THE REACTANTS.

The main object of this work was, however, to investigate whether compounds quite other than any possible reactants, *i.e.* neither esters, acids, nor alcohols, could influence the rate of hydrolysis by occupying part of the active surface of the enzyme.

Since the grouping common to all esters is —CO.O—, it is reasonable to consider that attachment of the ester to the active centre is achieved by some part of this group, either the carbonyl group or the ethereal oxygen atom. Ketones and ethers were therefore the first types of compounds investigated.

Effect of ketones.

The first ketone tried was acetone, but the inhibition in this case is very small. Increasing the length or weight of one of the chains, however, causes a great increase in the inhibiting powers. Thus methyl propyl ketone gives the following figures (see also Fig. 2):

Substrate: ethyl butyrate, 1 cc. Substrate and inhibitor present as emulsion.

1 cc. CO_2 in	56″	55″	53″	56″	*	1′ 45″	1′ 32″	1′ 51″	1′ 45″	1′ 53″	1′ 44″
2 cc. ,,	1′ 53″	1′ 55″	1′ 53″	1′ 53″	*	3′ 42″	3′ 23″	3′ 42″	3′ 31″	3′ 44″	3′ 40″
3 cc. ,,	2′ 53″	2′ 56″	2′ 54″	2′ 51″	*	5′ 33″	5′ 28″	5′ 36″	5′ 22″	5′ 35″	5′ 39″
4 cc. ,,	3′ 44″	3′ 48″	3′ 54″	3′ 46″	*	7′ 7″	7′ 17″	7′ 25″	7′ 3″	7′ 16″	7′ 24″

* 1 cc. substance added.

It is to be noted that all ketones give a true equilibrium with the substrate, and a linear course of hydrolysis in the mixture.

Acetophenone (methyl phenyl ketone) gives even greater inhibitions, thus, as seen from the following table, 0·1 cc. added to the normal quantity of reagents in the apparatus gives an inhibition of 33 %. (Inhibitions are expressed, after Rona, by the value $\frac{V_0 - V}{V_0} \times 100\ \%$, where V_0 = initial, V = final velocity.)

Substrate: ethyl butyrate, 1 cc. Substrate and inhibitor present as emulsion.

1 cc. CO_2 in	36″	34″	35″	*	51″	47″	51″	52″
2 cc. ,,	1′ 10″	1′ 8″	1′ 10″	*	1′ 46″	1′ 41″	1′ 45″	1′ 44″
3 cc. ,,	1′ 47″	1′ 46″	1′ 47″	*	2′ 39″	2′ 35″	2′ 40″	2′ 35″
4 cc. ,,	2′ 24″	2′ 23″	2′ 22″	*	3′ 34″	3′ 28″	3′ 37″	3′ 27″
5 cc. ,,	2′ 59″	3′ 0″	2′ 57″	*	4′ 28″	4′ 24″	4′ 40″	4′ 24″

* 0·1 cc. acetophenone added.

Quantitative relationships. Competitive adsorption.

The inhibitions obtained with acetophenone were so large that this substance was selected in order to work out the quantitative relationship. As stated above all the substances produce a stationary state or point of equilibrium, the velocity attaining and remaining at a constant value. The actual velocity depends on the relative concentrations of the two substances.

If the inhibitor acts in the way postulated, viz. is adsorbed by the enzyme in exactly the same way as is the substrate, then the inhibitor and substrate will compete for the available surface of enzyme. The following reasoning can then be applied.

At concentrations of the two substances greater than the saturation concentration (which can be experimentally found for the substrate and is known to be much lower than the experimental value), if c_1 and c_2 be the concen-

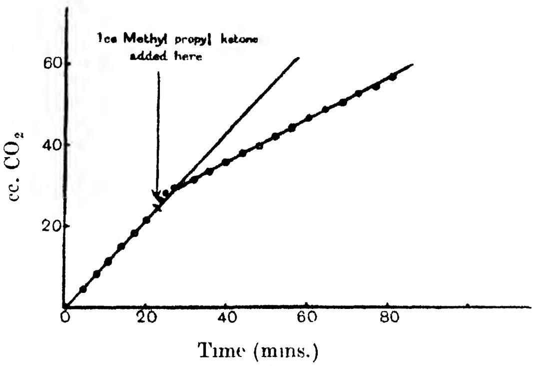

Fig. 2. Effect of methyl propyl ketone on hydrolysis of ethyl butyrate.

trations of the substrate S and inhibitor I respectively, and k_1 and k_2 their respective affinity constants, then $\frac{k_1c_1}{k_1c_1+k_2c_2}$ represents the fraction of the enzyme remaining active for purposes of hydrolysis. The velocity is directly proportional to this, so that V/V_0 is equal to this fraction. But the inhibition is defined

$$h = \frac{V_0 - V}{V_0} = 1 - \frac{k_1c_1}{k_1c_1 + k_2c_2} = \frac{k_2c_2}{k_1c_1 + k_2c_2}.$$

If for c_2/c_1 is written n, which is thus the number of mols of I per mol of S, and K for k_1/k_2, we have $h = \frac{n}{K+n}$. By this means K represents the value of n when the inhibition is 50 %.

Experimental values of h were found for several different relative concentrations of substrate and inhibitor and plotted. A theoretical curve was drawn for a value of K approximating to the mean of the values obtained. Fig. 3 shows the extent of agreement between the theoretical and observed values.

It was possible to obtain several points in a single experiment. V_0 was determined and a certain amount, 0·1 cc. in most experiments, of acetophenone then added through the top tap. When this V was satisfactorily obtained, that is when the velocity of gas evolution had become constant, a further quantity of the inhibitor or a further quantity of substrate could be added in the same way, and further points obtained. When additional substrate was given to the system containing a certain quantity of inhibitor a rise in the velocity was obtained. This in itself was sufficient to show that the

inhibitor did not act through any deleterious effect on the enzyme, but merely as a competitor with the substrate.

The extent of inhibition was moreover shown by experiment to be independent of the amount of enzyme present, but a function exclusively of the quantity here designated as n, that is the ratio of the concentrations of inhibitor and substrate. The two experiments quoted demonstrate this, the initial velocities which are proportional to the enzyme concentrations being approximately 2 to 3:

Substrate: ethyl butyrate.

A.

									Corrected value (by extrapolation) = 9′ 40″
						46″	*	50″	
1 cc. CO_2 in	51″	51″	*	1′ 8″	*	1′ 30″	*	1′ 41″	
					*	2′ 15″	*	2′ 37″	
2 cc. ,,	1′ 43″	1′ 43″	*	2′ 17″	*	3′ 2″	*	3′ 36″	
					*	3′ 49″	*	4′ 34″	
3 cc. ,,	2′ 36″	2′ 35″	*	3′ 28″	*	4′ 37″	*	5′ 35″	
					*	5′ 24″	*	6′ 31″	
4 cc. ,,	3′ 31″	3′ 24″	*	4′ 41″	*	6′ 14″	*	7′ 29″	
					*	6′ 58″	*	8′ 25″	
5 cc. ,,	4′ 25″	4′ 15″	*	5′ 53″	*	7′ 43″	*	9′ 23″	

Inhibitions—(i) 25 %; (ii) 43 %; (iii) 55 %.

B.

												Corrected value (by extrapolation) = 9′ 8″
									35″	*	50″	
1 cc. CO_2 in	33″	33″	*	40″	42″	*	53″	*	1′ 11″	*	1′ 39″	
								*	1′ 45″	*	2′ 31″	
2 cc. ,,	1′ 6″	1′ 6″	*	1′ 22″	1′ 26″	*	1′ 50″	*	2′ 21″	*	3′ 22″	
								*	2′ 57″	*	4′ 15″	
3 cc. ,,	1′ 40″	1′ 40″	*	2′ 2″	2′ 9″	*	2′ 46″	*	3′ 33″	*	5′ 8″	
								*	4′ 9″	*	6′ 2″	
4 cc. ,,	2′ 13″	2′ 12″	*	2′ 46″	2′ 54″	*	3′ 44″	*	4′ 45″	*	6′ 57″	
								*	5′ 19″	*	7′ 51″	
5 cc. ,,	2′ 46″	2′ 46″	*	3′ 30″	3′ 35″	*	4′ 42″	*	5′ 56″	*	8′ 47″	

Inhibitions—(i) 22 %; (ii) 40 %; (iii) 53 %; (iv) 70 %.

In the above two experiments the amount of ethyl butyrate at the commencement was 1·5 cc., and 0·1 cc. acetophenone was added at each *.

(The value of n is of course slowly changing throughout the experiment, as the concentration of substrate slowly diminishes while that of the inhibitor remains unaltered. The amount of substrate at any time is estimated from a knowledge of the rate of hydrolysis, *i.e.* of CO_2 output, and the time since the start of the experiment.)

Fig. 3 is drawn up from the following 22 sets of measurements, which in turn cover 8 separate experiments:

c_1	c_2	n (g. mol)	h	K	c_1	c_2	n (g. mol)	h	K
0·00674	0·00085	0·127	0·33	0·257	0·00674	0·00085	0·122	0·25	0·365
0·00674	0·00085	0·127	0·32	0·268	0·00673	0·00171	0·254	0·52	0·233
0·01030	0·00085	0·083	0·22	0·296	0·00673	0·00256	0·362	0·69	0·162
0·00701	0·00085	0·122	0·41	0·174	0·00986	0·00256	0·260	0·49	0·270
0·01080	0·00085	0·079	0·33	0·182	0·01030	0·00085	0·083	0·25	0·250
0·00674	0·00085	0·127	0·32	0·268	0·00994	0·00171	0·172	0·43	0·228
0·00606	0·00171	0·282	0·61	0·181	0·00986	0·00256	0·260	0·55	0·213
0·00945	0·00171	0·181	0·415	0·254	0·01030	0·00085	0·083	0·22	0·283
0·00701	0·00085	0·122	0·39	0·190	0·00994	0·00171	0·172	0·40	0·258
0·00631	0·00171	0·271	0·65	0·144	0·00986	0·00256	0·260	0·53	0·232
0·01012	0·00171	0·169	0·44	0·215	0·00945	0·00342	0·362	0·70	0·156

When the difficulty of accurately adding 0·1 cc. of a substance (which cannot be added in dissolved form) through the long side-tube projecting

above the water level in the bath and possessing a bend, is borne in mind, it will be appreciated that the extent of deviation observed is not more than the limit of experimental error.

The amounts of both acetophenone and ethyl butyrate used in the experiments exceed the solubility limits of the substances in water, and they are present in the system as an emulsion. The high speed of rotation of the stirrer however ensures that the emulsion is very fine and has the same composition all through except perhaps in the constriction leading to the exit tap. Furthermore the two substances are soluble in one another, so that the enzyme is presented with a uniform mixture of the two. The high speed

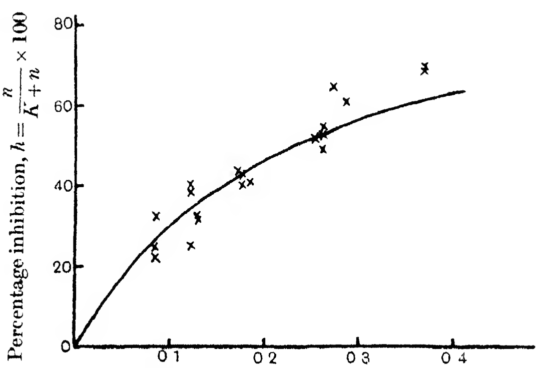

Fig. 3. Competitive adsorption between ethyl butyrate and acetophenone.
[The curve is the theoretical for $K = 0\ 23$].

of stirring ensures that mechanical access to the enzyme should not be dependent in any way on diffusion or any other factor of that nature. In conditions where that was the case these phenomena might well fail to be observed. The effect does not appear to be dependent on the amounts of the substances dissolved in the aqueous phase, for if that were so, increase of the concentration of either component above the aqueous solubility level would have no further effect on the velocity of hydrolysis, which is not the case. This must be especially emphasised, since a possible criticism of these results might be that effects attributed to chemical structure of substances were in reality merely due to greater or less solubility.

Comparison of competitive adsorption with effect of fluoride.

It is interesting at once to contrast this type of inhibition, which we have termed "competitive adsorption," with a quite different type, namely that brought about by fluoride. On chemical grounds it would not be expected that fluoride would be likely to compete with the substrate, and quantitative experiments show that the behaviour of this substance is in fact quite different.

Thus the experiment cited in the accompanying table shows that after addition of fluoride with resulting fall of the velocity to a new constant level, a further addition of substrate will not raise the level again as with competitive adsorption types. The inhibition is thus not dependent on the relative concentration of inhibitor to substrate at all. But it is dependent on the relative concentration of inhibitor to enzyme. Whereas in cases of competitive adsorption the enzyme concentration does not affect the value of the percentage inhibition, in this case, the less enzyme the more is the percentage inhibition caused by a given quantity of fluoride. Fluoride in fact behaves as if it formed a compound with the enzyme itself, which no amount of substrate will reverse. Yet this compound is not irreversible since Loevenhart and Peirce [1906] showed that after dialysis a completely inactivated sample regains its activity, and quantitative relationships confirm this. No exact quantitative experiments were included in this series, but it was shown that if x, say, was the amount of fluoride required to give 50 % inhibition, *i.e.* to inactivate half of the enzyme, then $2x$ would not suffice to inactivate the whole, as it would were the combination irreversible.

Pancreatic lipase is very sensitive to $M/40$ fluoride (1 in 1000), this concentration giving an inhibition of nearly 90 % with the amount of enzyme employed in these experiments, while $M/40$ chloride would have no appreciable effect. One part of fluoride in 7000 gives an inhibition of 25 % in these conditions.

Showing non-competitive inhibition of fluoride.

1 cc. CO_2 in	46″	45″	*	57″	59″	†	1′ 1″	1′ 1″
2 cc. ,,	1′ 30″	1′ 31″	*	1′ 57″	1′ 58″	†	2′ 7″	2′ 1″
3 cc. ,,	2′ 16″	2′ 13″	*	2′ 58″	2′ 59″	†	3′ 10″	3′ 2″
4 cc. ,,	3′ 2″	2′ 57″	*	4′ 0″	4′ 3″	†	4′ 11″	4′ 2″
5 cc. ,,	3′ 50″	3′ 42″	*	5′ 1″	5′ 6″	†	5′ 14″	5′ 0″

* 1 cc. 1 % sodium fluoride solution added.

† 0·5 cc. ethyl butyrate added.

Effect of other molecular structures.

The experiments with acetophenone, and worked out to a less extent with other ketones, *e.g.* methyl propyl ketone, lead to the conclusion that ketones are adsorbed by the active centres of lipase, and compete with the normal substrates for the surface of the centres. It was next necessary to investigate to what extent other compounds exerted an influence on the velocity.

Effect of ethers.

As stated before, the possibility exists that the esters could be adsorbed partially by the ethereal oxygen atom, and ethers would then act as inhibitors. Anisole (methyl phenyl ether), was particularly tried since the groups flanking the oxygen are the same as those present in acetophenone. It was found, as the table shows, that in comparison with the effect of the latter, the inhibition given by anisole is very feeble:

Showing the effect of anisole on hydrolysis of ethyl butyrate, 1 cc. Substrate and inhibitor present as emulsion.

1 cc. CO_2 in		32″	33″	30″	*	40″	35″	41″
2 cc.	,,	1′ 5″	1′ 7″	1′ 2″	*	1′ 19″	1′ 10″	1′ 20″
3 cc.	,,	1′ 37″	1′ 40″	1′ 35″	*	1′ 57″	1′ 46″	1′ 57″
4 cc.	,,	2′ 8″	2′ 12″	2′ 7″	*	2′ 36″	2′ 21″	2′ 33″
5 cc.	,,	2′ 39″	2′ 48″	2′ 41″	*	3′ 13″	3′ 1″	3′ 9″

* 0·1 cc. anisole added here.

$$\text{Inhibition } h = \frac{V_0 - V}{V_0} = 1 - \frac{V}{V_0} = 1 - \frac{t_0}{t} = 13\ \%.$$

A similar volume of acetophenone gives an inhibition of 33 %.

Some controls. Hydrocarbons.

As experiments showed that a wide variety of compounds had some small amount of inhibitory power, as indeed is not unexpected, it was thought desirable to make a fairly wide survey, particularly including aromatic derivatives, since these in general gave greater inhibitions. It is to be expected that hydrocarbons, having very inert molecules and no polar groups (besides being insoluble) would exert no action at all. Pentane and hexane among aliphatic, benzene, toluene, ethylbenzene and cyclohexane among cyclic hydrocarbons, were investigated. Pentane, hexane and benzene had no final effect whatever, an immediate rise in the velocity of gas evolution lasting a few minutes being followed by a permanent return to the original level. As much as 1 cc. of benzene in 70 cc. reacting fluid has been used with no effect. Toluene and ethylbenzene act as very weak inhibitors: no reason is known for this phenomenon. Very frequently, but not always, if more than 0·1 cc. were used, no steady state set in, but a gradual diminution of velocity was observed. Cyclohexane was unique in producing a permanent activation. The cause was not discovered, but presumably must be physical rather than chemical in nature. Presence of the substance did not affect the power of other substances to inhibit, *e.g.* acetophenone was able to bring about its usual inhibition substituting the new value for V_0. The activation was equally marked in the case of completely soluble substrates such as triacetin, so that the effect, whatever it may be, appears to be on the enzyme itself.

Some soluble aromatic derivatives.

More soluble benzene derivatives were tried, *e.g.* phenol and aniline. 0·1 g. phenol in the total content gives an inhibition of 15 %, whereas 0·1 g. acetophenone gives over 30 % inhibition, in spite of the fact that phenol is considerably more soluble. This again points against the criticism that the effect is merely one of relative solubility. Aniline, which is fairly soluble, gives a very weak inhibition. Two experiments, each using 0·1 g. of this substance, have given inhibitions of 5·5 % and 6·5 % respectively. This highly polar substance therefore does not seem to be very actively adsorbed by the enzyme.

The following two tables give the experiments the results of which have

been quoted. The substance was suspended in bicarbonate solution, and added at the points marked *:

Substrate: ethyl butyrate.

A. Effect of 0·1 g. phenol.

1 cc. CO_2 in	40″	36″	35″	*	48″	49″	41″
2 cc. ,,	1′ 20″	1′ 12″	1′ 10″	*	1′ 27″	1′ 33″	1′ 25″
3 cc. ,,	1′ 58″	1′ 49″	1′ 47″	*	2′ 5″	2′ 20″	2′ 8″
4 cc. ,,	2′ 35″	2′ 26″	2′ 23″	*	2′ 47″	3′ 2″	2′ 52″
5 cc. ,,	3′ 11″	3′ 3″	3′ 3″	*	3′ 33″	3′ 46″	3′ 36″

Inhibition = 15 %.

B. Effect of 0·1 g. aniline

1 cc. CO_2 in	35″	32″	30″	*	34″	32″	34″	33″
2 cc. ,,	1′ 7″	1′ 3″	1′ 0″	*	1′ 5″	1′ 4″	1′ 6″	1′ 5″
3 cc. ,,	1′ 36″	1′ 33″	1′ 29″	*	1′ 37″	1′ 36″	1′ 40″	1′ 39″
4 cc. ,,	2′ 5″	2′ 5″	1′ 59″	*	2′ 8″	2′ 9″	2′ 13″	2′ 11″
5 cc. ,,	2′ 36″	2′ 36″	2′ 30″	*	2′ 44″	2′ 40″	2′ 48″	2′ 43″

Inhibition = 5·5 %.

Effect of phenylmethylcarbinol.

Further experiments designed to show that the very powerful inhibitory action of acetophenone is due to its carbonyl group consisted in investigating derivatives in which this group was no longer present. The first compound investigated was phenylmethylcarbinol. Since alcohols are reactants in the general lipase system, this substance might be expected to be adsorbable by the lipase, and the results are in agreement with this supposition. 0·1 g. of this substance gives an inhibition of 25 % when the ethyl butyrate present is 1 cc. (0·88 g.), or about three-fourths of the inhibition given by acetophenone. When quantitatively worked out, the inhibition given by this substance was found to conform to the competitive adsorption type, the gram-molecular affinity relative to ethyl butyrate being about 2·5–3 for this compound as against about 4–5 for acetophenone. (These figures represent $1/K$ for the substances.) This shows that the alcohol does not give rise to any appreciable back action although being powerfully adsorbed. The case is analogous to the effect of glucose and fructose on the hydrolysis of cane-sugar by invertase. They occupy the surface of the enzyme.

The following table represents a typical result with this substance:

Effect of phenylmethylcarbinol on hydrolysis of ethyl butyrate, 1 cc. Substrate and inhibitor present as emulsion.

1 cc. CO_2 in	42″	41″	*	52″	53″	54″	*	1′ 12″	1′ 11″
2 cc. ,,	1′ 22″	1′ 22″	*	1′ 48″	1′ 48″	1′ 50″	*	2′ 31″	2′ 28″
3 cc. ,,	2′ 5″	2′ 4″	*	2′ 44″	2′ 44″	2′ 46″	*	3′ 48″	3′ 49″
4 cc. ,,	2′ 46″	2′ 45″	*	3′ 39″	3′ 41″	3′ 41″	*	5′ 8″	5′ 7″
5 cc. ,,	3′ 27″	3′ 29″	*	4′ 34″	4′ 38″	4′ 37″	*	6′ 24″	6′ 28″

Inhibitions = (i) 25 %: (ii) 46·5 %.

* 0·1 g. phenylmethylcarbinol added.

Whether it is the same active centre for the adsorption of the ester and of the alcohol is still not clear. In the dehydrogenases the groupings —CO— and —CH.OH— seem interchangeable, *i.e.* either is equally well adsorbed at the same centre. It is not clear whether this is the same effect or whether phenylmethylcarbinol acts merely as an alcohol. In this case the ketone

exerts a more powerful action than the corresponding alcohol, and a keto-acid, as will presently be described, had more effect than a hydroxy-acid, but with the pair of substances, cyclohexanone and cyclohexanol, the reverse was true. Both, in comparison with the pair, acetophenone and phenylmethylcarbinol, are very weakly adsorbed, but cyclohexanol is very definitely more adsorbed than cyclohexanone, as the following two experiments show:

Substrate: ethyl butyrate 1 cc. Substrate and inhibitor present as emulsion.

A. Effect of cyclohexanone.

1 cc. CO_2 in	37″	37″	*	43″	42″	41″	†	49″	49″	‡	47″	46″
2 cc. ,,	1′ 13″	1′ 13″	*	1′ 25″	1′ 24″	1′ 21″	†	1′ 38″	1′ 39″	‡	1′ 32″	1′ 32″
3 cc. ,,	1′ 51″	1′ 52″	*	2′ 7″	2′ 6″	2′ 3″	†	2′ 28″	2′ 31″	‡	2′ 17″	2′ 15″
4 cc. ,,	2′ 29″	2′ 32″	*	2′ 49″	2′ 48″	2′ 45″	†	3′ 19″	3′ 20″	‡	3′ 5″	3′ 0″
5 cc. ,,	3′ 8″	3′ 10″	*	3′ 31″	3′ 29″	3′ 27″	†	4′ 10″	4′ 10″	‡	3′ 53″	3′ 43″

* 0·5 cc. added. † Another 0·5 cc. added. ‡ Another 0·5 cc. ethyl butyrate added.

B. Effect of cyclohexanol.

1 cc. CO_2 in	38″	38″	*	50″	54″	50″	†	46″	45″	‡	1′ 5″	1′ 0″
2 cc. ,,	1′ 14″	1′ 15″	*	1′ 37″	1′ 50″	1′ 42″	†	1′ 32″	1′ 30″	‡	2′ 8″	2′ 0″
3 cc. ,,	1′ 52″	1′ 52″	*	2′ 27″	2′ 45″	2′ 34″	†	2′ 17″	2′ 15″	‡	3′ 8″	3′ 0″
4 cc. ,,	2′ 30″	2′ 31″	*	3′ 17″	3′ 43″	3′ 27″	†	3′ 3″	3′ 1″	‡	4′ 10″	4′ 0″
5 cc. ,,	3′ 8″	3′ 8″	*	4′ 8″	4′ 38″	4′ 22″	†	3′ 49″	3′ 48″	‡	5′ 12″	5′ 1″

* 0·5 cc. added. † Another 0·5 cc. ethyl butyrate added. ‡ Another 0·5 cc. inhibitor added.

The gram-molecular constants ($1/K$) for these substances are approximately 0·17 for cyclohexanone and 0·6 for cyclohexanol.

Effect of acetophenoneoxime.

A more satisfactory attempt to affix the properties of acetophenone to its carbonyl group was in the use of the oxime. A difficulty admittedly arises in that this substance is a solid, but it is fairly soluble, and other solids have been investigated with which to compare it. The observed effect with solids is probably less than the full, as the solid tends to sink to the bottom but if very finely powdered, nearly all the material is kept evenly distributed throughout the fluid by the stirrer.

Among solids used have been benzophenone, benzoin, and benzil. Two experiments with the former have yielded, for 0·1 g. of the substance, inhibitions of 15 % and 17 %, corresponding to a gram-molecular constant of 2·5, less than the observed value for acetophenone, but this may be accounted for by steric hindrance, the position of the carbonyl group between two rings quite possibly presenting an obstacle to adsorption. Benzoin has given figures of about the same order, but the error here is probably greater as the substance appears more prone to aggregate and fall to the bottom. Benzil, which, being a diketo-compound, is of especial interest, is particularly prone to this objection, the finely powdered material aggregating to large lumps as soon as it enters the fluid, nevertheless a considerable inhibition has been observed.

With these, then, it was considered fair to compare acetophenoneoxime. Whilst at first it was suspected of having some residuary inhibiting power, repeated recrystallisation finally gave a product giving practically no inhibition at all.

The following table compares acetophenoneoxime with benzophenone.

Substrate: ethyl butyrate, 1 cc. Inhibitors present as suspensions.

A. Effect of 0·1 g. benzophenone, added at *.

1 cc. CO_2 in	35″	36″	36″	*	42″	40″	39″
2 cc. ,,	1′ 9″	1′ 12″	1′ 12″	*	1′ 22″	1′ 20″	1′ 18″
3 cc. ,,	1′ 44″	1′ 49″	1′ 48″	*	2′ 4″	2′ 1″	2′ 0″
4 cc. ,,	2′ 20″	2′ 25″	2′ 24″	*	2′ 45″	2′ 43″	2′ 42″
5 cc. ,,	2′ 55″	3′ 0″	3′ 0″	*	3′ 27″	3′ 24″	3′ 25″

Inhibition = 15 %.

B. Effect of 0·1 g. acetophenoneoxime, added at *.

1 cc. CO_2 in	34″	34″	*	36″	35″	36″
2 cc. ,,	1′ 5″	1′ 8″	*	1′ 12″	1′ 9″	1′ 7″
3 cc. ,,	1′ 37″	1′ 43″	*	1′ 47″	1′ 44″	1′ 42″
4 cc. ,,	2′ 14″	2′ 17″	*	2′ 23″	2′ 19″	2′ 17″
5 cc. ,,	2′ 53″	2′ 53″	*	2′ 59″	2′ 55″	2′ 53″

No inhibition.

Miscellaneous examples.

Benzaldehyde also gives an inhibition of an order approximating to that given by acetophenone, the inhibition by 0·1 cc. in the usual volume being 26·5 %, corresponding to a gram-molecular constant ($1/K$) of 2·75. The effect of the carbonyl group is thus again evident. The oxime of this substance has not been investigated.

The behaviour of ethyl benzoate should not pass unnoticed. Preliminary experiments had shown that this ester was quite unattacked by the lipase preparation used. Further experiments showed that the substance acted as a simple inhibitor, not a very powerful one, but displaying competitive inhibition when added to a system actively hydrolysing ethyl butyrate or other substrate.

In connection with the experiments using methyl propyl ketone, acetylacetone was investigated. This substance, containing as it does two keto-groups, might be expected to have high inhibitive properties. Actually it was not so active as methyl propyl ketone itself. This is probably due to the fact that it is actively tautomeric, with a labile hydrogen atom, and does not therefore function as a ketone.

Effect of pyruvate and lactate.

Lastly remain to be described some experiments in which the grouping to be investigated was part of the anion of a salt. The sodium salt of a keto-acid, pyruvic, was compared with the similar salt of the corresponding hydroxy-acid, lactic acid, while the unsubstituted propionic acid was used as a control. The necessary concentrations of these entirely soluble substances were much larger than those of the compounds previously considered, amounts of the order of 1 g. being used. In the experiments quoted 1 g. each of pyruvic and lactic acids (whose molecular weights differ only by 2) and the corresponding equimolecular weight of propionic acid, all neutralised with sodium hydroxide, were used. With pyruvate it was very difficult to obtain the value

[1] The results with solids other than benzophenone have not been included in the general table, as for reasons stated they are not capable of strict comparison with the other results. This does not affect the view that the comparison just drawn represents a true qualitative difference of behaviour between the substances.

of the final velocity, as there tended to be a gradual decline in this quantity, but the inhibition was in the zone from 30 to 40 %. With lactate the inhibitions observed varied from 18 to 22 %, while propionate gave 11 %; equimolecular sodium chloride gave 8 %, while the dilution error (for these substances had to be introduced in a volume of at least 5 cc.) was up to 5 %. The relative effects of these substances are shown graphically in Fig. 4.

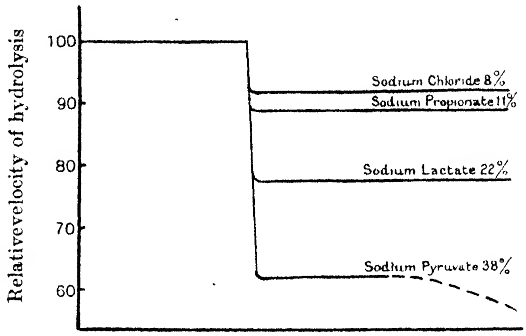

Fig. 4.

Incidentally the approximation between the values given by sodium propionate and sodium chloride shows that the fatty acids, present as they are as ions at this p_H, are very weakly adsorbed. This agrees with the statement made at the beginning of this paper that the continuance of the linear curve of hydrolysis depended on the alcohol produced, and scarcely at all on the acid.

Finally, Fig. 5 represents graphically the relative extent of the inhibition produced by the different substances studied. It shows the diminution in velocity brought about by a concentration of one-tenth of a gram-molecule of the inhibitor per gram-molecule of ethyl butyrate.

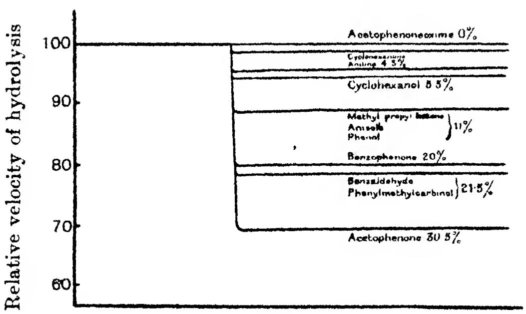

Fig. 5.

The values of 1/K relative to ethyl butyrate (= 1) for a number of chemically related substances are appended:

	1/K		1/K
Acetophenone	4·35	Phenol	1·25
Benzophenone	2·5	Aniline	0·45
Benzaldehyde	2·75	Cyclohexanol	0·60
Phenylmethylcarbinol ...	2·75	Cyclohexanone	0·17
Anisole	1·25		

Conclusion.

Whilst the nature of the active centres of lipase is still quite unknown, except in so far as we know from the work of Willstätter and his colleagues that they are not protein in nature, the work described throws some light on the manner of the attachment of the substrate to those active centres. Thus if substances containing the carbonyl group compete with the substrate for the enzyme, it may be claimed to show that it is by this group that the substrate is normally attached, which makes the relative non-specificity of the enzyme more understandable. The conversion of —CO— into —CHOH still yields a substance with inhibitive power, but in no case has it been found that replacement of —CO— by —CH_2 results in an active inhibitor. It is clear however that the non-polar moieties attached to the —CO— group also play an important part in rendering the substance accessible to the enzyme, as is indicated for instance by the greater inhibition by methyl propyl ketone than by acetone. The actual affinity of the enzyme for the substrate (the Michaelis constant), and the readiness of decomposition of the resulting complex are determined by factors in which the nature and configuration of the rest of the molecule play the chief part, and with regard to which lipases of different origin differ among themselves. The varying sensitivity of the different lipases to poisons such as alkaloids, arsenicals, etc., which has been much studied and which is held to be independent of the state of purity, is again a different phenomenon. The work described is meant to bear on the general affinities between substrates and the active centres of the lipase, the general rather than the particular having been the object of study. The results observed support the conclusions concerning the nature of enzyme action which have been obtained from the study of the dehydrogenating enzymes of bacteria.

Summary.

1. A method is described by which the actual velocity of hydrolysis of esters by lipase can be followed immediately.

2. The effects of various compounds, studied from the point of view of their molecular structure, on this velocity is described.

3. Ketones (including aldehydes) produce an inhibition of the velocity of lipase hydrolysis out of proportion to that produced by the chemically related compounds investigated. Secondary alcohols also produce an inhibition, which may however be due to reversal of hydrolysis.

4. This power is completely lost by converting the ketone into its oxime.

5. It is claimed that this is due to a specific affinity between the carbonyl group and the active centre of the enzyme, which affinity is also responsible for the normal adsorption of esters preparatory to their hydrolysis by the enzyme.

In conclusion I wish to express my thanks to Sir F. G. Hopkins for his constant interest in this work, and to Mr J. B. S. Haldane and Dr J. H. Quastel for continued help and advice. I am also indebted to the Department of Scientific and Industrial Research for a supplementary grant.

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XXXVIII. THE USE OF DECINORMAL HYDROCHLORIC ACID FOR STANDARDISING ELECTROMETRIC p_H MEASUREMENTS.

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In his specifications for the standardisation of p_H measurements, Clark [1928] gives as the ultimate standard the tenth-normal calomel half-cell, to which is assigned the Sørensen [1909] value for the normal hydrogen electrode potential. As a secondary standard any solution of known p_H may be used, the term p_H being defined with reference to the first standard.

The practical application of these specifications is a matter of some difficulty. The tenth-normal calomel cell is not now widely used owing to the amount of labour required for its satisfactory preparation; in addition to the tedious purification of the chemicals it is necessary to make up several cells at a time and take the average potential which must be frequently checked against freshly made cells [Sorensen and Lindestrom-Lang, 1924]. If the alternative method is used, the materials for the working saturated calomel cell need not be specially purified, but it is difficult to find a satisfactory standard solution.

Of the many buffer solutions whose p_H has been determined, none is suitable even for rough measurements unless the salt is recrystallised several times. The 0·05 M potassium hydrogen phthalate recommended by Clark and Lubs [1916] is the simplest to prepare and has undoubtedly been successfully used by many workers, but the presence of impurity is a possible source of danger. Thus it is noted by Clark [1928] that the best commercial phthalic anhydride, although usually satisfactory, sometimes contains impurities which necessitate ten or more recrystallisations of the salt prepared from it; in the absence of an independent standard this precaution would not be taken. The occasional presence of another very troublesome impurity is described in a note at the end of this paper. Most of the other buffer solutions available are even more difficult to prepare and are not often employed for the present purpose.

A solution of the composition 0·01 N HCl.0·09 N KCl is advocated by Sørensen and Lindestrøm-Lang [1924] and others, but there appears to be some uncertainty both in the p_H and the temperature coefficient [Clark, 1928; Cullen, Keeler and Robinson, 1925]. Decinormal hydrochloric acid has none

of these disadvantages, as the pure acid is readily obtainable and the 0·1 N solution easily standardised. It has been used by Cullen [1922] and many others [Cullen, Keeler and Robinson, 1925; Hastings and Sendroy, 1924; Hastings, Baird, Sendroy, Julius and Van Slyke, 1928] who accept the value of 1·08 for the p_H at all temperatures. Since this value is based on the hydrogen ion activity calculated from the E.M.F. measurements of Noyes and Ellis [1917], it gives to the procedure a theoretical basis, the advantage of which has been pointed out by Cullen, Keeler and Robinson. There remains, however, the problem of the potential at the liquid junction

$$0{\cdot}1\ N\ \mathrm{HCl/sat.\ KCl}$$

which is certainly much greater than that produced between saturated KCl and any of the other proposed standard solutions. It is, of course, usual to neglect the value of this potential, but it is still necessary that the junction should be formed in a reproducible manner. It has been shown by Clark and Lubs [1916] that the drift of potential which occurs at such junctions as this is considerable, and interferes with the accuracy of the measurement, and Clark [1928] considers that while each worker will be able to reproduce his own results, there is a serious danger of discrepancy between the results of different workers if this solution is used as a standard. The present paper is chiefly concerned with a practical study of the liquid junction in question from the standpoint of Clark's criticism.

Experimental.

The potential difference of the cell

$$+\mathrm{Hg/HgCl.}0{\cdot}1\ N\ \mathrm{HCl}\underset{A}{/}\mathrm{sat.\ KCl}\underset{B}{/}3{\cdot}5\ N\ \mathrm{KCl.HgCl/Hg}-$$

was measured, junction A being made by a variety of methods. No importance is to be attached to the absolute value of the voltages recorded, as the chemicals were not specially purified, but the results show that the potentials at the electrodes remained unchanged during each series. The calomel vessels used were similar to those described by Kerridge [1926], and were kept at 25° by means of a simplified air-bath [Clark, 1928]. The temperature of the air in the bath varied by about 0·1° every half-minute, but this fluctuation produced no measurable effect on the readings obtained. Junctions A and B were made with vertical glass tubes dipping into the saturated KCl which was contained in a small intermediate vessel. Evaporation was prevented by a layer of liquid paraffin.

Saturation of the KCl was ensured by using a solution saturated at 37°, which was allowed to come to 25° and then well stirred. Junction B was always made at the end of a tube of 3 mm. internal diameter; its contribution to the P.D. of the cell was probably negligible. Junction A was formed both inside and at the end of tubes of different diameters. In the former case it was made by pinching the rubber tube, which held the glass tube in which the junction was to be established, in order to expel two or three drops of

HCl into a waste vessel when the glass tube was dipped into the KCl and the pressure released; a sharp junction was thus drawn into the tube about 1 cm. from the end (see Fig. 3). With wide tubes (7 mm.) it was found necessary to use a Y-piece and rubber teat. In practice, instead of the cell being lowered, the KCl was raised and a support finally pushed underneath. It was necessary to dislodge the drop of liquid paraffin which sometimes adhered to the end of the tube before drawing the junction inside. The following table shows typical results obtained with one pair of half-cells.

Table I.

(*a*) Junction at end of tube of 7 mm. internal diameter							
Reading (mv.)	84·8	84·6	84·4	84·2	84·0	83·8	83·7
Time (min.)	1	3	6	11	19	35	54
(*b*) Junction at end of 3 mm. tube							
Reading (mv.)	84·5	83·9	83·65	83·35	83·2	83·1	
Time (min.)	1	6	12	42	76	138	
(*c*) Junction inside 7 mm. tube							
Reading (mv.)	84·9	84·75	84·75				
Time (min.)	1	10	60				
(*d*) Junction inside 3 mm. tube							
Reading (mv.)	84·9	84·75	84·75				
Time (min.)	1	10	60				
(*e*) Junction inside 1 mm. tube							
Reading (mv.)	85·1	84·9	84·8				
Time (min.)	1	10	60				

It will be noticed that there is a surprising difference between junctions formed inside the tube and those formed at the end. The former are much to be preferred for the following reasons.

(*a*) They are more reproducible.

(*b*) The time change is only about one-tenth as great and occurs almost wholly within the first ten minutes.

(*c*) The diameter of the tube is unimportant if 3 mm. or over.

The final readings (10 minutes or longer) in Table I (*c*) and (*d*) were the most reproducible. The order of accuracy obtainable is shown by the following series of consecutive readings (obtained with another pair of cells) for junctions inside 3 mm. tubing. Each reading represents a different junction 10 minutes or longer after forming.

84·84, 85·00, 84·85, 84·95, 84·93, 84·94, 84·97, 84·90, 85·00, 84·90, 84·85, 84·90, 84·95 mv.
Av. deviation = 0·04 mv.; max. deviation = 0·08 mv.; mean: 84·92 mv.

None of the other readings was reproducible to less than about ± 0·2 mv. This type of junction was compared with a flowing junction by means of the apparatus sketched in Fig. 1.

The lighter HCl enters through tube *C* and meets an upward stream of the heavier KCl in the broad tube *D*. The mixed stream overflows at *E*. The junction can be made or broken as often as desired by raising or lowering tube *C* which fits the cork somewhat loosely. *C* and *D* are connected with reservoirs of HCl and KCl with rubber tubing, the rate of flow being regulated with screw clips. A T-piece is included on each side for connecting with the

two calomel cells. A convenient rate of flow is about 20 drops per minute from E, and the P.D. then reaches almost immediately a constant value which is unaffected by large increases in the rate of flow of either solution, or by the alteration of the diameter of tube C from 3 mm. to 1 mm. The exact degree of reproducibility is uncertain as the galvanometer was only sensitive to 0·05 mv. with the greater resistance introduced. No variation could be detected over periods of a quarter of an hour. Static junctions were made inside tube C in the usual way.

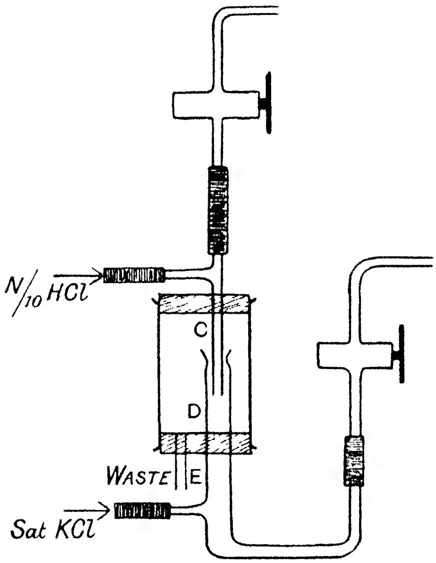

Fig. 1.

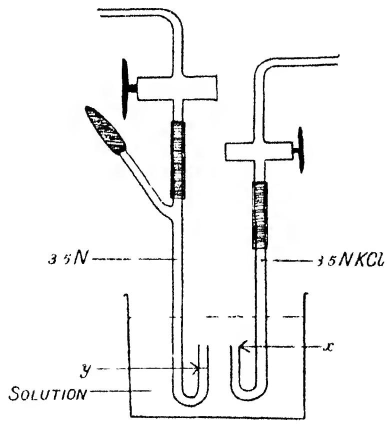

Fig. 2.

This form of flowing junction appears to possess certain advantages over the possibly more reproducible types of Lamb and Larson [1920] and McInnes and Yeh [1921], since contact can be made or broken as often as desired and the rate of flow of each solution can be varied independently. It was designed to imitate the first flowing junction described by Lamb and Larson, where the lighter solution runs slowly into a large volume of the heavier. Another pair of cells was used which, with the flowing junction at A, gave a potential difference of 88·9 mv. at the beginning and at the end of the experiment. This value fell to about 87·6 mv. when the flow of HCl was stopped, and rose to about 90·4 mv. when the flow of KCl was stopped. The average final reading of five static junctions made inside tube C was 88·89 mv. Taking into account the sensitivity of the galvanometer, it is evident that the two methods agree to within 0·05 mv.

The junction between saturated KCl and some other solutions was studied at room temperature with the arrangement shown in Fig. 2, using the system

$$\mathrm{Hg/HgCl}.3{\cdot}5\,N\ \mathrm{KCl/solution}/3{\cdot}5\,N\ \mathrm{KCl.HgCl/Hg}.$$
$$\qquad x \qquad\qquad\qquad y$$

Junction y was first made inside the tube with the aid of a rubber teat and after 10 minutes junction x was made at the end of the tube. The pair of cells used for these experiments gave a P.D. of $<$ 0·05 mv. before and after the series. The following results are typical of those obtained.

Table II.

0·1 N HCl					
Reading (mv.)	+0·6	+0·7	+0·85	+0·98	+1·10
Time (min.)	1	3	6	13	19
0·01 N HCl.0·09 N KCl					
Reading (mv.)	+0·17	+0·26	+0·28		
Time (min.)	0·5	5	10		
Potassium hydrogen phthalate 0·05 N					
Reading (mv.)	−0·06	+0·06	+0·07		
Time (min.)	1	7	10		
Phosphate solution p_H 7·4					
Reading (mv.)	+0·05	+0·10	+0·14	+0·16	
Time (min.)	1	4	7	19	

The difference between the two types of junction is not very serious except in the case of 0·1 N HCl.

The above system was also used to test the reproducibility of saturated KCl agar bridges and of the ground glass junctions used by Kerridge [1926] with the glass electrode: these were substituted for the tube shown at junction x. The agar bridges were prepared as directed by Michaelis and Fujita [1923] in tubing of 1 mm. internal diameter. The two cells used here had a P.D. of ± 0·5 mv. before and after the experiment.

Table III.

Agar bridge with 0·1 N HCl[1]						
Reading (mv.)	+1·0	+1·5	+1·7	+2·0	+2·5	(±0·5 mv.)
Time (min.)	1	3	9	20	60	
Agar bridge with phosphate solution						
Reading (mv.)	+0·5	+0·8	+0·9	(±0·5 mv.)		
Time (min.)	1	5	15			
Glass caps with HCl						
Reading (mv.)	−20	−10	−4	−6	−1	−1·5
Time (min.)	1	3	10	1	3	15
Glass caps with phosphate solution						
Reading (mv.)	−1·5	0	+1	(±0·5 mv.)		
Time (min.)	1	5	20			

It will be seen that with the phosphate buffer both methods gave results corresponding roughly with the junction inside the tube, the order of accuracy being about ± 0·5 mv. The same is true of agar bridges with 0·1 N HCl, but the glass caps are unsuitable for making contact with this solution. In using the ground glass caps it was essential to exclude air bubbles, which may cause large errors. This is best accomplished by running some KCl solution into the cap as it is placed in position; the outside can then be rinsed and dried with filter paper as usual.

[1] The correct reading here is about 1·5 mv., since junction y was formed with 3·5 N i .ction of saturated KCl (see Table IV). .orming

Discussion.

In the case of static junctions it has always been the practice to take the initial readings, largely on the recommendation of Cumming and Gilchrist [1913]. The junctions have sometimes been at the ends of tubes [Fales and Vosburgh, 1918; Sørensen and Lindestrøm-Lang, 1924; Harned, 1926] and sometimes inside tubes [Clark, 1915; Stern, 1925], but even in the latter case the results have not been satisfactory for HCl solutions. For example, Clark and Lubs [1916], using 0·01–0·05 N HCl/sat. KCl, report a maximum deviation from the mean of 0·22 mv., and an average deviation of 0·14 mv. These results presumably correspond to the initial readings in Table I (c) and (d), which showed about the same variation. McInnes and Yeh [1921] also note that the initial reading differs from that given by their flowing junction in the case of 0·1 N HCl/0·1 N KCl. It is significant that Cumming and Gilchrist favoured initial readings chiefly on the evidence afforded by junctions made at the end of the tube; actually they record a negligible time change in the case of junctions inside a 1 cm. tube.

Since the absolute value of the potential at the liquid junction is unknown, all that is required is a reproducible result. It is therefore suggested that for the present purpose it is better to take the final reading (10 minutes or longer) with a junction made inside a tube of 3 mm. or over by the technique described above. Even with 0·1 N HCl, the degree of reproducibility is satisfactory for most purposes, $\pm$ 0·05 mv. or $\pm$ 0·001 p_H, and the value obtained in this case is very close to that given by the flowing junction, and probably within 0·3 mv. of the value given by initial readings with the Clark hydrogen electrode. Final readings could no doubt be taken with this electrode, and should be identical with those obtained by the above method. The modification of Bunker's [1920] electrode as sketched in Fig. 3 is simpler and was found to be quite suitable for checking buffer solutions. It saturates quickly and, if provided with taps, the current of hydrogen may be turned off for at least half an hour without affecting the potential, which shows that the protection from the air is adequate. With this electrode there is no difficulty in duplicating results on 0·1 N HCl to within 0·1 mv.

It is claimed that this treatment of the liquid junction will remove any danger in the use of 0·1 N HCl for the standardisation of p_H measurements, and that this is, therefore, the safest solution available for this purpose. At the same time, the justice of Clark's criticism, referred to above, is clearly shown by the results obtained. Thus for the solutions 0·1 N HCl/sat. KCl the initial readings for junctions at the end of the tube may differ from those obtained with the above method by 0·3 mv., and probably from those usually obtained with the Clark electrode by 0·6 mv.; in addition to this they are less reproducible on account of the greater time change, a delay of even one or two minutes in taking the reading being serious. Agar bridges or capillary tubing give rise to errors of one or two millivolts, and ground glass junctions are ommended.

The "elimination" of the liquid junction potential.

There are two empirical methods which aim at the elimination of the liquid junction potential; the Bjerrum [1911] extrapolation method, and that of Michaelis and Fujita [1923]. The latter consists in interposing a solution of 0·1 N HCl saturated with KCl thus:

$$+\,\mathrm{Hg/HgCl.0{\cdot}1}\ N\ \mathrm{HCl}\underset{A}{/}0{\cdot}1\ N\ \mathrm{HCl.sat.\ KCl}\underset{B}{/}3{\cdot}5\ N\ \mathrm{KCl.HgCl/Hg}\,-$$

The two half-cells used in the first experiment (84·92 mv.) were compared by both these methods with the following results, which apply to final readings inside 3 mm. tubes.

Michaelis and Fujita	... ...	90·4 mv.
3·5 N KCl 83·8; 1·75 N KCl 79·9	Bjerrum's point	87·7 mv.

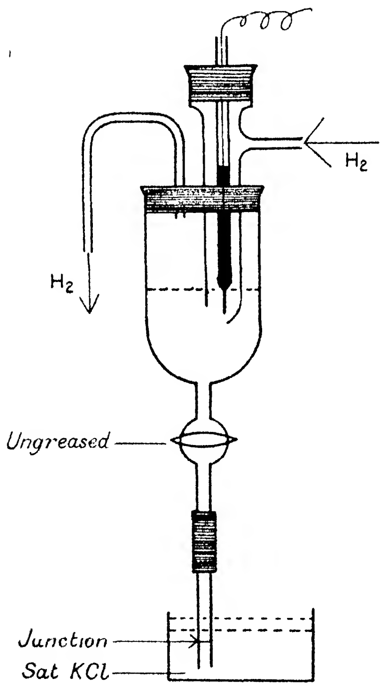

Fig. 3.

The two results differ widely and, therefore, do not support the validity of either method for this junction. Somewhat better agreement is obtained by following the practice of Walpole [1914] and Clark and Lubs [1916], who apply the Bjerrum extrapolation, determined substantially as above, to the voltage developed when saturated (4·1 N) KCl is used as a bridge. This gives 88·8 mv. for Bjerrum's point. It was noted in using the method of Mich[illegible] and Fujita that the time change was in the opposite direction t[illegible]nction observed in all the other cases. This time change was almost entir[illegible] forming

junction A, since remaking junction B, when A had attained equilibrium, produced a negligible (< 0·05 mv.) change in the voltage. This observation suggested that some intermediate strength of HCl would eliminate the time change, and the following results (Table IV) show that the required strength is about 0·0185 N. The values obtained for Nos. 4 and 5 were very close, and as they were not reproducible to less than ± 0·1 mv. they overlapped. The average has, therefore, been taken as representing the mixture sat. KCl-0·0185 N HCl with a very small time change of variable sign. This difficulty was probably due to slight initial diffusion which has a relatively great effect on these junctions.

Table IV.

The P.D. of the cell

$$+\mathrm{Hg/HgCl.0{\cdot}1}\ N\ \mathrm{HCl}\underset{A}{/}\mathrm{solution}\ x\underset{B}{/}3{\cdot}5\ N\ \mathrm{KCl.HgCl/Hg}\ -$$

No.	Solution x	Final reading, inside junction mv	Average time change mv.
1	1·75 N KCl	79·9	−0·5
2	3·5 N KCl	83·8	−0·3
3	Sat. (4·1 N) KCl	84·9	−0·2
4	Sat. KCl.0·0183 N HCl	86·15 av.	−0·1*
5	Sat. KCl.0·0188 N HCl		+0·1*
6	Sat. KCl.0·085 N HCl†	90·4	+1·0
7	Sat. KCl.1·0 N HCl	119·0	+1·2

* These figures refer to junctions at the end of the tube; the time change for inside junctions was negligible in these cases.

† This mixture is 0·1 N HCl shaken with solid KCl to saturation, as directed by Michaelis and Fujita [1923].

The significance of this nul point is doubtful, but a consideration of Table IV suggests the possibility that the liquid junction potential has been eliminated in this case. In the first place it is probable on theoretical grounds that the liquid junction potential at A in No. 3 is small. The reading which corresponds to elimination of this potential is then not more than a few millivolts positive to 84·9. Thus, possibly in No. 6 and certainly in No. 7, the potential has been reversed. It is quite probably reversed in No. 6 since Harned [1916] has shown that the hydrogen ion activity of 0·1 N HCl is doubled by the presence of 3 M KCl. Now the amount of time change is frequently considered to be an index of the liquid junction potential. In Nos. 1, 2, 3 and 7, it is undoubtedly an index both of the sign and magnitude of the potential; if the same be true of Nos. 4, 5 and 6, it follows that the potential at A is zero in Nos. 4 and 5. This argument ignores the effect of junction B which, however, showed no appreciable time change even in the case of No. 7. In order to obtain some estimate of the potential at this junction, which cannot be zero, the Bjerrum extrapolation was used thus:

duc

at inute'g/HgCl.0·1 N HCl $\underset{A'}{/}$ $\begin{matrix}0{\cdot}0185\ N\ \mathrm{HCl}\\ \mathrm{sat.\ KCl}\end{matrix}$ $\underset{B'}{/}$ $\begin{matrix}\mathrm{sat.\ KCl}\ (86{\cdot}20)\\ 3{\cdot}5\ N\ \mathrm{KCl}\ (86{\cdot}15)\\ 1{\cdot}75\ N\ \mathrm{KCl}\ (85{\cdot}95)\end{matrix}$ / 3·5 N KCl.HgCl/Hg −

give.

omme

This gives the P.D. at B' (with 3·5 N KCl) as $-$ 0·2 mv. No accuracy can be claimed for this value, but it is probably of the right order of magnitude. On these assumptions the potential at the flowing junction

$$- 0{\cdot}1\ N\ \mathrm{HCl/sat.\ KCl} +$$

is 1·45 mv. Two recently calculated values are 1·58 mv. [Harned, 1926] and 4·7 mv. [Scatchard, 1925]. It is doubtful, however, whether the P.D. at A' can really be zero, as the hydrogen ion activity is different on the two sides of the junction. The p_H of the solution 0·0185 N.HCl sat. KCl was measured with the hydrogen electrode; assuming 1·08 for the p_H of 0·1 N HCl and neglecting liquid junction potentials the p_H of the mixture was found to be 1·32.

A note on the use of potassium hydrogen phthalate.

A commercial sample of "pure" potassium hydrogen phthalate contained less than 0·1 % water and required the theoretical volume of 0·1 N NaOH for neutralisation when titrated as directed by Dodge [1915]. Yet the p_H of the 0·05 N solution was 3·92 instead of 3·97. This was found to be due to the presence of a trace of impurity, presumably the acid salt mentioned by Dodge [1920]. This impurity was sparingly soluble in cold water, more soluble in hot, so that after four recrystallisations, carried out above 20°, the resulting product would not dissolve completely in water at ordinary dilution.

50 g. of the original material were shaken with 100 cc. water and allowed to stand, when the clear solution was decanted. This process was repeated until an apparently insoluble residue was obtained. 0·5016 g. of this residue was dissolved in hot water and required 26·60 cc. 0·1 N NaOH for neutralisation instead of the theoretical 24·54 cc.

The impurity might be introduced during the preparation either by an error of neutralisation or by recrystallisation below 20° [see Dodge, 1920], and, as it cannot be removed by recrystallisation and may not be obvious at a dilution of 0·05 N, it represents a danger which should not be overlooked. In view of this, and of Clark's note on phthalic anhydride mentioned above, it is suggested that if potassium hydrogen phthalate is to be used as a standard the only safe course is to recrystallise until the p_H of the 0·05 N solution is unaffected by further recrystallisation (above 20°). This can be done without using an independent standard and is not very laborious if the original material is pure; samples such as that described above are of course quite unsuitable, but at least the presence of the impurity is revealed by this treatment.

Summary.

1. The advantages of using decinormal hydrochloric acid for standardising p_H measurements are pointed out. The liquid junction requires special treatment.

2. Some of the factors influencing the P.D. at the liquid junction 0·1 N HCl/sat. KCl have been investigated and a simple method of forming

the junction is described, which is reproducible to ± 0·05 mv. or ± 0·001 p_H. This method is also recommended for general use in accurate p_H determinations.

3. A new type of flowing junction is described which gives for these solutions results almost identical with those obtained by the above method.

4. The degree of reproducibility of junctions formed with agar bridges and with ground glass surfaces is considered.

5. Some observations on the time change at the junction 0·1 *N* HCl/sat. KCl.*xN* HCl are presented and the possibility of eliminating the potential at this junction is discussed.

6. The occurrence of an impurity in a certain sample of potassium hydrogen phthalate is described. This affected the p_H of the 0·05 *N* solution but could not be removed by recrystallisation.

In conclusion I wish to express my thanks to Prof. E. C. Dodds for his advice and criticism. The expenses of the investigation were defrayed by Mr Poupart's gift to the Dental Research Fund, on whose behalf the work was undertaken.

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XXXIX. SCYLLITOL IN SELACHIAN ONTOGENY.

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One of the principal aims of chemical embryology must certainly be to find out, in the case of any given egg, exactly what the embryo is provided with by the maternal organism. Anything with which it is not provided at the start must either be absorbed from the environment or synthesised during development, and it is important to know how much of this kind of work the embryo has to perform during its morphogenesis. We know, for example, that the eggs of marine invertebrates are not supplied with enough water or enough ash to make the complete embryo, while those of fishes have enough ash but not enough water. With regard to organic constituents also the same distinction can be made. As far as we know all eggs contain sufficient protein, and in no case does protein have to be manufactured from other nitrogenous bodies. Probably the same holds true of carbohydrate, but undoubtedly certain fishes and gastropods have to synthesise fatty acids during their ontogeny, and there is evidence that in the eggs of birds, for instance, some 15 % of the total cholesterol which the embryo requires has to be formed from other substances. Lipins, on the other hand, judging from the behaviour of ether-soluble phosphorus, are always present in ample amount at fertilisation, though the range of species for which we have information is not large. The two most outstanding instances of substances which are not provided by the maternal organism are pigments—thus no egg contains haemoglobin—and cycloses.

In a previous paper [Needham, 1924] it was shown that the *i*-inositol which is found in all the tissues of the newly hatched chick has to be synthesised by the embryo during the three weeks of its development. This observation had been previously made by Klein [1909] but the course of formation of the cyclose by the chick was followed by an estimation-method, which, though it left much to be desired, was an improvement on any of the techniques which had been at the disposal of the earlier workers.

The inositol of birds and mammals is not the only one which occurs in animal tissues. Of its seven possible stereoisomeric modifications, one other is known, scyllitol, first isolated from elasmobranch fishes by Stadeler and Frerichs [1858]. This body is identical with the quercinitol which has been obtained from acorns, and with the cocositol of the palm-tree [Müller, 1912]. In view of the fact that the cycloses have recently acquired considerable

physiological importance through the identification of *i*-inositol with (Wildier's) Bios I by Eastcott [1928], and for the general embryological reasons already mentioned, it was thought worth while to investigate the origin of scyllitol during the development of a selachian fish. The material, collected at the Millport Marine Biological Station, consisted of eggs and embryos of *Acanthias vulgaris* (the "spur-dog") and *Scyllium canicula* (the "rough-dog").

The method of isolation of the scyllitol embodied the most useful parts of various previous methods. The eggs or minced embryos were preserved in acetone and, when the time came, were extracted repeatedly with acetone. The combined extracts were then freed from acetone by a vacuum distillation and the aqueous solution extracted several times with ether in order to remove pigments and substances of a fatty nature. Most of the pigment of the eggs and some of the pigment from the embryos was thus removed. The solution was freed from ether by evaporation and protein precipitated by boiling with acetic acid at a p_H at which bromocresol purple was just green. A saturated solution of neutral lead acetate was then added after neutralisation and the precipitate, which was usually small in amount, filtered off and discarded. To 100 parts of the filtrate were now added 60 parts of a saturated solution of basic lead acetate, 20 parts of a 40 % solution of cadmium nitrate, and 5 parts of concentrated ammonia, for the cycloses are completely carried down on the precipitate of lead hydroxide and the double salt of cadmium and lead which is formed. The precipitate was then filtered off, washed, suspended in distilled water, and decomposed with hydrogen sulphide. The lead sulphide was next removed by filtration, three or four extractions with water at 70° being required to wash the cyclose completely away from it. A second treatment with hydrogen sulphide was sometimes necessary, but, if not, the solution was evaporated down to about 10 cc. and poured into 20 times its volume of absolute alcohol, when the scyllitol separated in the form of a microcrystalline powder. After being allowed to stand overnight it was filtered off and weighed, a correction being made for whatever ash the product contained, as the cycloses, being insoluble in all organic solvents, are rather difficult to free from salts.

In some cases the simpler method of Rosenheim [1917] was used, in which the original extracts, freed from acetone, and allowed to stand at a low temperature, deposit crystals of scyllitol. A sample of scyllitol prepared in the course of this work melted at 330°. The following results were obtained.

Acanthias vulgaris:

Exp. B_1. 8 *yolks of undeveloped eggs* (the perivitelline liquid, which was included, is very small in amount):

Total weight, 167 g.; *i.e.* 20·8 g. each egg wet weight.
Total dry weight, 53 g.; *i.e.* 6·63 g. each egg dry weight.
Percentage of water, 68·2.
Scyllitol isolated, 2·0 mg.;
i.e. 0·25 mg. per egg;
1·2 mg. per 100 g. wet weight;
.00 g. dry weight.

Exp. D. 3 *yolks of undeveloped eggs:*

Total wet weight, 75·7 g.; *i.e.* 25·2 g. each wet weight.
Total dry weight, 33 g.; *i.e.* 11 g. each dry weight.
Percentage of water, 56·5
Scyllitol isolated, traces only, insufficient for weighing.

Exp. A_m. 2 *yolks of 23 cm. embryos:*

Total wet weight, 37 g.; *i.e.* 18·5 g. each wet weight.
Total dry weight, 12·6 g.; *i.e.* 6·3 g. each dry weight.
Percentage of water, 66.
Scyllitol isolated, 12·0 mg.;
i.e. 6·0 mg. per egg;
32·4 mg. per 100 g. wet weight;
95·1 mg. per 100 g. dry weight.

Exp. E. 2 *embryos of 23 cm. length* (corresponding to the twentieth month of development out of twenty-one [Ford, 1921]):

Total wet weight, 80 g.; *i.e.* 40·0 g. each wet weight.
Total dry weight, 12·5 g.; *i.e.* 6·25 g. each dry weight.
Percentage of water, 84·1.
Scyllitol isolated, 52·5 mg.;
i.e. 26·25 mg. per embryo;
65·7 mg. per 100 g. wet weight;
420·0 mg. per 100 g. dry weight.

Exp. F. 12 *embryos of 23 cm. length:*

Total wet weight, 496 g.; *i.e.* 41·3 g. each wet weight.
Total dry weight, 61 g.; *i.e.* 5·09 g. each dry weight
Percentage of water, 87·6.
Scyllitol isolated, 292·2 mg.;
i.e. 24·3 mg. per embryo;
59·0 mg. per 100 g. wet weight;
480·0 mg. per 100 g. dry weight.

Exp. G. *Adult muscle:*

From 47·75 kg. wet weight (9·55 kg. dry weight) 5·75 g. of scyllitol were obtained; this yield was almost exactly the same as that obtained from 50 kg. by Rosenheim [1917] and Müller [1912]: 0·012 mg. per 100 g. wet weight; 0·06 mg. per 100 g. dry weight.

Scyllium canicula:

Exp. C. 4 *egg-contents (yolk and jelly):*

(As *Acanthias vulgaris* is an ovoviviparous form, retaining its eggs and embryos within the uterus until the end of their development, it might be argued that the supply of scyllitol was derived from the mother. *Scyllium*, however, is oviparous.) Dry weight, 4·36 g.; *i.e.* 1·09 g. dry weight each egg. Scyllitol isolated; traces only, insufficient for weighing.

The material used in the above experiments was not very abundant, for n
it is somewhat difficult to collect, but the results are quite concordant an m-
permit of the conclusion that the scyllitol present in the embryos at the e hese
of development has to be synthesised by them and is not provided in
egg at the beginning. The dogfish is thus in exactly the same position a
chick. In Fig. 1 are shown graphically the relations which have been
to hold in the two kinds of eggs. Eastcott [1928] determined the an
i-inositol present in undeveloped hen's eggs by a biological assay d
on the identification of inositol with Bios I, and, as the diagram
results agree very well with the figures obtained in direct an
interesting to note that scyllitol itself will not function as Bi
private communication).

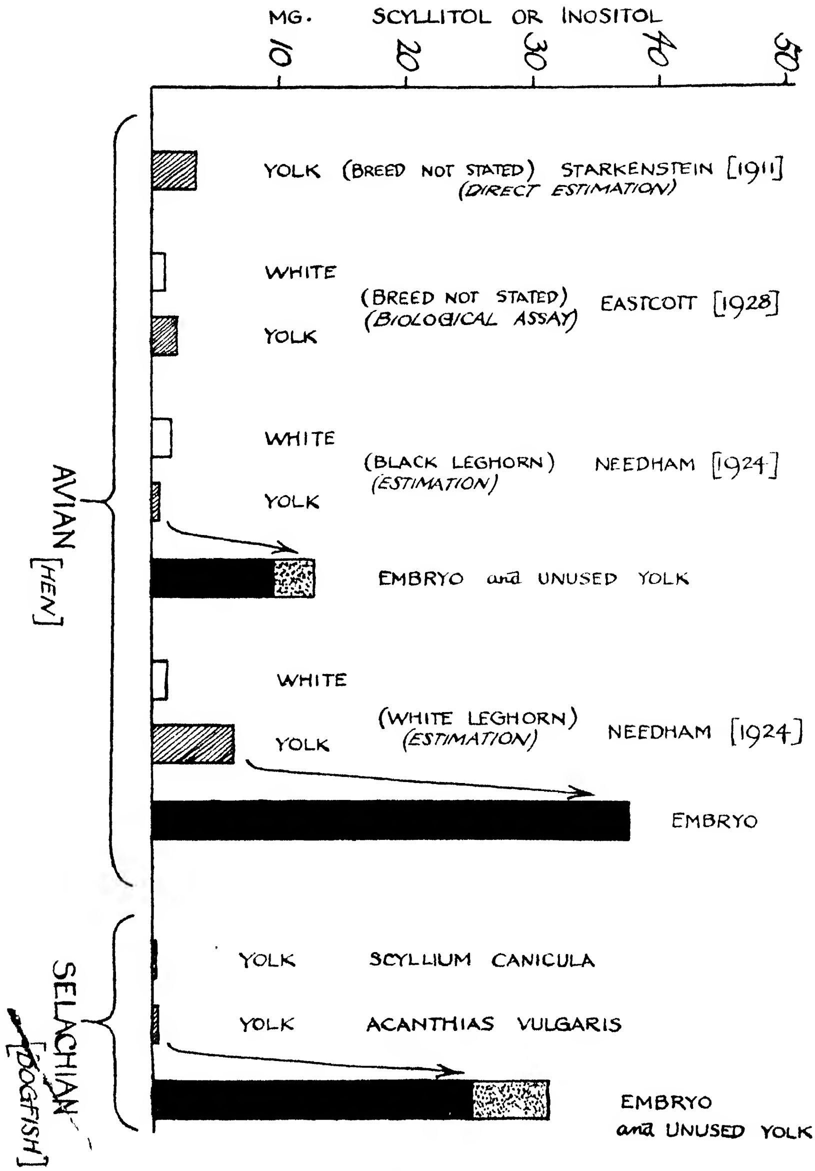
MG. SCYLLITOL OR INOSITOL
10
20
30
40
50
YOLK (BREED NOT STATED) STARKENSTEIN [1911]
(DIRECT ESTIMATION)
WHITE
(BREED NOT STATED)
(BIOLOGICAL ASSAY)
EASTCOTT [1928]
YOLK
WHITE
(BLACK LEGHORN)
(ESTIMATION)
NEEDHAM [1924]
YOLK
EMBRYO and UNUSED YOLK
WHITE
(WHITE LEGHORN)
(ESTIMATION)
NEEDHAM [1924]
YOLK
EMBRYO
AVIAN [HEN]
YOLK SCYLLIUM CANICULA
YOLK ACANTHIAS VULGARIS
EMBRYO
and UNUSED YOLK
SELACHIAN [DOGFISH]

Fig. 1.

One further point which emerges from the data given above may be mentioned. The distribution of solid and water is evidently different in the embryos from what it is in the eggs, and the facts may be written as follows:

		Water	Solid
		g.	g.
Acanthias vulgaris:	Undeveloped eggs	14·2	8·8
	Finished embryos	35·0	6·0

This is another illustration of the rule that in fishes the egg is not provided with sufficient water to make the embryo, but has to absorb it from the environment, and, as it were, to dilute the raw materials with it. No doubt in the case of *Acanthias*, where the eggs remain within the maternal body, the water required is supplied from the maternal blood, but this dogfish stands low in the Ercolani placenta classification, for it has nothing but pits and inequalities on the maternal side, pressed up against the smooth horny egg-case. Little else than water, therefore, probably passes between maternal blood and the eggs.

The present communication adds further to the evidence that cyclose can be synthesised by animal cells. To the two cases of its formation in the egg may be added its formation by polyuric rats on pure diet, and by the plant, as has recently been shown by Eastcott for yeast. It would be interesting to know whether the eggs of lamellibranchiate gastropods contain enough mytilitol for the needs of the embryo or not.

Summary.

The scyllitol of the undeveloped eggs and fully developed embryos of the selachian fish, *Acanthias vulgaris*, was investigated. It was found that just as the chick has to synthesise during its ontogeny 90 % of the inositol with which it hatches, so the dogfish has to synthesise 90 % of its scyllitol. The cycloses would therefore seem not to be provided in the eggs of birds or fishes by the maternal organism.

The writer's thanks are due to Mr Richard Elmhirst and the staff of the Millport Marine Biological Laboratory whose kind assistance and co-operation made the work possible. He is also indebted to the Government Grant Committee of the Royal Society for a grant which covered the cost of these researches.

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XL. NOTE ON ANAEROBIOSIS AND THE USE OF ALKALINE SOLUTIONS OF PYROGALLOL.

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Effective precautions are usually taken to remove poisonous impurities from hydrogen used in securing anaerobiosis. It is difficult to find in bacteriological literature references to the need for recognising that a poisonous gas, carbon monoxide, is evolved when atmospheric oxygen is absorbed by alkaline solutions of pyrogallol. Wright [1928], following Burrows [1917, 1921] in his method of preparing pure nitrogen from commercial cylinder nitrogen, took precautions to ensure that no carbon monoxide, resulting from the small amount of oxygen present, should pass over into the anaerobic chamber.

The author, in conjunction with Drakeley [1925, 1929], has shown that all alkaline solutions of pyrogallol, of strengths likely to be used in practice, evolve carbon monoxide when oxygen of high purity is absorbed. With cylinder oxygen of about 98 % purity, amounts from 0·1 % up to practically 10 % of carbon monoxide may be formed, and absence of agitation of the absorbent solution favours the production of larger amounts of carbon monoxide than those formed when the solution is vigorously shaken. Oxygen of lower purity, such as atmospheric oxygen, liberated carbon monoxide in all cases investigated, though the percentage evolution was smaller.

These findings have a bacteriological import, since the conditions under which alkaline solutions of pyrogallol are used in bacteriology do not usually favour agitation of the solution during absorption of oxygen. A similar remark may be made with reference to some pomological investigations, as, for example, examination of the course of ripening of fruits in the absence of oxygen.

It was also shown that all alkaline solutions of pyrogallol absorb oxygen, though with varying degrees of efficiency. There appears consequently to be no warrant for the practice of some authors of specifying with exactness a particular concentration of alkali, while leaving vague the amount of pyrogallol.

The method of Rockwell, as quoted by Stitt [1927], is of much interest. Rockwell calls for the use of sodium bicarbonate solution with addition of sodium dihydrogen phosphate, according to the formula:

Sodium bicarbonate	50 g.
Sodium dihydrogen phosphate	0·75 g.
Water	500 cc.

"Keep tightly corked."

In use, "0·5 g. pyrogallic acid and 1 cc. of the charged alkali solution" are mixed *in situ* (culture tube).

This might seem to be an attempt to avoid production of carbon monoxide, but it appears to be an omnibus method for producing carbon dioxide in replacement of the absorbed oxygen. As such, its chemistry is complicated. A qualitative study of a solution prepared according to Rockwell's direction was made. It was found that at room temperature a solution of pyrogallol in sodium bicarbonate solution did not appreciably absorb oxygen whether the acid phosphate were present or not. The amount of bicarbonate specified did not entirely dissolve. A roughly quantitative evolution of carbon dioxide took place when the acid phosphate was added to the bicarbonate solution. When the solution was warmed in an incubator at 38°, oxygen was slowly absorbed, owing to bicarbonate becoming partly transformed into normal carbonate; carbon dioxide was given off, partly as a product of that transformation, and partly from the interaction of acetic acid (an oxidation product of pyrogallol) with the carbonates.

A quantitative study was attempted, though, owing to the incomplete solubility of the components at room temperature, it was thought desirable to vary the quantities. 50 g. of pyrogallol were dissolved in 50 cc. of solution prepared according to the above formula, the solution being then made up to 200 cc. with access of air. After standing overnight at room temperature in a corked bottle, the solution was transferred to a Hempel pipette: thus free carbon dioxide was lost. The solution was treated with four successive amounts of commercial cylinder oxygen of about 98·5 % purity and it was allowed to stand in each case without agitation for periods of 24 or 72 hours in a thermostatically controlled water-bath at 38°. Results of analyses of the residual gases are given in the table.

No. of exp.	Volume of cylinder oxygen cc.	Hours in thermostat (approx.)	Percentage of unabsorbed oxygen	Percentage of carbon dioxide	Percentage of carbon monoxide
1	96·0	72	0·05	64	0·55
2	94·7	24	1·4	36	0·7
3	98·0	24	1·4	1·4	0·8
4	100·0	24	3·7	49	1·0

The percentage of carbon dioxide was variable, and depended largely upon the length of time which had elapsed between the moment the pipette was removed from the thermostat and the time analysis was begun. In Exp. 3 the pipette was allowed to stand 24 hours at room temperature before making analysis of the gases; the carbon dioxide was almost entirely absorbed during cooling.

When measured volumes of air were admitted into the pipette, which was then placed in the thermostat, it was found that the final volume of gas was always greater than the initial volume, owing to production of an amount of carbon dioxide greater than the volume of oxygen absorbed. A similar increase of volume was found when the pipette was filled with inert gases

(nitrogen and argon) left from a previous analysis of air. When the pipette was allowed to stand empty of gas, no gas was produced. It was evident that carbon dioxide was produced during the reversible thermal transformation of bicarbonate into carbonate, the acid phosphate taking no part in this production after the initial evolution, of which the effect is lost when the "charged solution" is mixed with pyrogallol before use.

No accurate figures can be given for the percentage of carbon monoxide evolved when this modification of Rockwell's solution was used as absorbent for atmospheric oxygen, owing to the fact that the gases were measured in a simple Hempel burette, and not in the Bone and Wheeler apparatus which was used in the earlier work. In four analyses of air, a small and just detectable amount of carbon monoxide was produced from about 100 cc. of air; since the limit of measurement of the burette readings was 0·05–0·1 cc., this indicated an evolution of carbon monoxide of the order of 0·3 %, calculated on the oxygen absorbed. The result was in agreement with analyses made, using solutions of pyrogallol in caustic potash.

It would seem desirable that the effect of small amounts of carbon monoxide upon the growth of micro-organisms should be investigated, since even such a weakly alkaline solution of pyrogallol as the modification of Rockwell's solution used in this work, is capable of evolving carbon monoxide when the solution is used as an absorbent of oxygen. In conclusion, it may be said that there is little evidence for the idea of combination between pyrogallol and alkalis in aqueous solution: the term "pyrogallate solution" should be abandoned in favour of "alkaline solution of pyrogallol".

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XLI. SEED FATS OF THE UMBELLIFERAE.

II. THE SEED FATS OF SOME CULTIVATED SPECIES.

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Attention has been directed in earlier communications [Hilditch and Jones, 1928; Hilditch, 1928] to the presence of $\Delta^{6:7}$-octadecenoic acid or petroselinic acid in the fatty oil of parsley and other seeds of the Umbelliferae. It was shown in the preceding paper of this series that the seed fats of two native wild species of the order, *Heracleum Sphondylium* and *Angelica sylvestris*, contain in each case about 20 % of this isomeric oleic acid, together with 44–50 % of oleic, 25–33 % linoleic and about 4 % of palmitic acid. Seven other instances of Umbellate seed fats, drawn from common cultivated varieties, have now been examined and petroselinic acid has been found to be present in considerable amount in each case, the proportion varying from 26–60 % of the total mixed fatty acids.

It is therefore possible to state with some confidence that $\Delta^{6:7}$-octadecenoic acid is characteristic of seed fats of the Umbelliferae. The presence of this acid has not been reported, moreover, in fats originating from any other botanical order with the sole exception of the Araliaceae (a closely allied Umbellate family). (It may be mentioned that, since our former communications, a further analysis of the seed fat of *Hedera Helix* has been published by Steger and van Loon [1928], who state that in this case 55 % of the mixed fatty acids consists of petroselinic acid.)

In the present work we have followed our usual procedure as far as possible, in order to obtain a quantitative measure of the various fatty acids present. The mixed fatty acids from the saponified fats were treated with lead acetate in alcoholic solution and a preliminary separation was thus effected into (1) saturated acids, petroselinic acid, and probably a little ordinary oleic acid, and (2) oleic and linoleic acids, possibly accompanied by traces of palmitic and petroselinic acids. The separation of ordinary oleic acid from petroselinic acid by this method is not likely to be completely quantitative, and therefore to that extent the results of the work must be considered of an approximate nature. The separation is, however, sufficiently marked to permit a reasonably accurate estimate of the content of petroselinic acid in the various fats to be obtained.

Each group of acids was then converted into methyl esters and the latter submitted to fractional distillation at a very low pressure.

Another complication, from the point of view of quantitative work, was the presence of non-fatty matter (frequently of a resinous character) in many of the ester fractions; this rendered the interpretation of the saponification equivalents of the latter doubtful in many cases. The same difficulty was encountered in the investigation of *Heracleum* and *Angelica* fats [Hilditch and Jones, 1928], and our experience confirms that of other workers who have noted that Umbelliferous seed fats are prone to be accompanied by unusually large amounts of non-fatty material both in the form of essential oils and of resinous compounds.

Although as much volatile essential oil as possible was removed by heating each fat in a vacuum prior to its hydrolysis, one or two instances were met with (notably, the seed fats of fennel and carrot) in which there remained some volatile compound of an unsaponifiable nature which eventually came over in the more volatile fractions of the distilled methyl esters.

Again, the saponification equivalents of the main ester fractions from certain of the fats (notably celery and parsnip) were sometimes erratic, apparently owing to small proportions of resinous (? lactonic) compounds which tended to distil along with the methyl esters.

The residues from the methyl ester distillations of course contained comparatively large amounts of non-volatile unsaponifiable matter, which, especially by reason of its dark colour, made the determination of mean saponification equivalents of these fractions extremely difficult.

Qualitative examination of the fatty acids present in the methyl ester fractions obtained indicated in all cases that the acids present were only palmitic, petroselinic, oleic and linoleic[1]; no saturated acid lower than palmitic was detected, and the hexabromide test failed to disclose the presence of any linolenic acid in any of the specimens examined. Consequently the numerical calculations have been based wherever possible on the observed iodine values rather than the equivalents, except that in those fractions containing large amounts of unsaponifiable matter (that is, the residual non-volatile fractions and certain of the lowest boiling fractions in the cases of fennel, carrot and caraway) the proportion of unsaponifiable matter has been determined from the saponification equivalent figures.

Experimental.

The seed fats investigated were extracted by means of carbon tetrachloride from the ground dried seeds. We are much indebted to Mr T. E. Miln, of Messrs Gartons Ltd., who very kindly procured for us supplies of the various

[1] The methods employed in these investigations have not disclosed up to the present the occurrence of a diethylenic acid structurally related to $\Delta^{6,7}$-octadecenoic acid rather than to $\Delta^{9,10}$-octadecenoic acid; but the possibility of the existence of such an acid should not be overlooked.

seeds and ensured that the material handled was of definite botanical origin. The crude seed fats obtained by the solvent extraction (in amounts varying from 350–950 g.) had the characteristics shown in the following table.

Table I.

			Characteristics of seed fat				
						Unsaponifiable matter	
Species	Common name	Fat content of seed %	Saponification equivalent	Acidity (as % oleic acid)	I.V.	Non-volatile	Volatile (up to 150°/1 mm.)
Foeniculum capillaceum Gilib.	Fennel	13·3	309·4	16·9	87·9	11·3	5·0
Daucus Carota Linn.	Carrot	15·9	307·7	8·0	106·4	13·25	0·9
Coriandrum sativum Linn.	Coriander	8·8	289·6	28·6	89·5	9·27	1·1
Apium graveolens Linn.	Celery	14·4	291·8	5·7	129·1	2·9	15·2
Pastinaca sativa Linn.	Parsnip	17·3	272·1	12·9	92·7	2·6	—
Chaerophyllum sativum Gartn.	Chervil	11·3	295·3	1·7	139·4	3·3	—
Carum Carvi Linn.	Caraway	9·0	300·9	9·9	112·1	0·9	13·9

The volatile essential oils removed by vacuum treatment of the heated crude fats were not further investigated, since they had no bearing on the immediate purpose of the research. The non-volatile unsaponifiable matter was not removed from the fatty acids prior to further treatment, but was left to accumulate in the residues from the ester distillations.

The crude seed fats (after removal of volatile matter as described) were saponified with excess of alcoholic potash, and the mixed crude fatty acids isolated and dried.

The fatty acids (1 part) were dissolved in boiling 95 % alcohol (5 parts) and mixed with a boiling solution of lead acetate (0·7 part) in 95 % alcohol (5 parts). After boiling for a few minutes the mixture was set aside overnight at room temperature and the separated lead salts were removed by filtration next morning. The insoluble lead salts were recrystallised from a volume of 95 % alcohol equal to that employed in the original separation, and separated by filtration after standing at room temperature overnight. The recrystallised insoluble lead salts were converted back to the fatty acids, and are referred to below as "solid" acids. The mother liquors from the recrystallisation were (except in the case of parsnip seed fat) united with the original alcoholic filtrates, and the recovered acids therefrom are denoted as "liquid" acids. In the case of the parsnip fatty acids the "liquid" acids from the two alcoholic filtrates were worked up separately.

The yields of the two groups of acids from the various seed fats are shown in Table II.

Each group of fatty acids obtained (1 part) was separately dissolved in methyl alcohol (2 parts) and heated under reflux in presence of 2 or 3 % of sulphuric acid for 2 hours; this effected almost quantitative conversion into

the corresponding methyl esters which, after removal of most of the excess of methyl alcohol, were taken up in ether and washed with dilute sodium carbonate solution until neutral. The crude neutral methyl esters were heated in a vacuum at about 100° to remove the last traces of solvent and were then fractionally distilled.

Table II.

	"Solid" acids		"Liquid" acids	
Seed fat	%	I.V.	%	I.V.
Fennel	60·2	83·1	39·8	110·2
Carrot	57·1	87·0	42·9	145·5
Coriander	59·6	79 8	40 4	104 7
Celery	54·0	84·8	46·0	119·5
Parsnip	46·2	87·6	21·7	128·2
			32 1	119 0
Chervil	57·5	100·7	42 5	175 7
Caraway	28·7	80·2	71 3	127 5

The results of the methyl ester separations are summed up in the next series of tables, together with the calculated composition of the individual fractions, determined in the majority of cases (as already stated) from the iodine values in conjunction with the data obtained by qualitative examination of individual ester fractions.

For those fractions which contained notable amounts of unsaponifiable matter it was essential to utilise in addition the saponification equivalents: in these instances the soap solutions from the saponified esters were thoroughly extracted with ether, and the saponification equivalent and iodine value of the recovered fatty acids (freed from unsaponifiable matter) were then determined. These values calculated back to the corresponding methyl esters are (when necessary) inserted in the tables between brackets.

For purposes of identification, individual ester fractions were hydrolysed and the resulting soaps oxidised by means of dilute ice-cold alkaline permanganate solution according to the directions of Lapworth and Mottram [1925]. The liberated mixture of dihydroxy- and tetrahydroxy-stearic acids was boiled with light petroleum (B.P. 40–50°) to effect the removal of any saturated fatty acids; the latter (only palmitic acid was detected) were purified by crystallisation from aqueous alcohol. The crude hydroxystearic acids were boiled with water in order to remove any sativic acid and then crystallised from ethyl acetate.

Petroselinic acid was readily identified in the form of the corresponding 6 : 7-dihydroxystearic acid, which melts at 121–122°. The 9 : 10-dihydroxy-acid from ordinary oleic acid, which melts at 130°, was used to define the presence of $\Delta^{9:10}$-oleic acid. In all cases the identity of the compounds was checked by determination of mixed melting point with authentic specimens of the respective acids.

In several cases a dihydroxystearic acid (or quite possibly an equilibrium mixture of acids) was encountered which melted consistently at 113–115°.

This substance was only met in oxidation products from the "liquid" esters and it is possible that it indicates the presence, in the acids with soluble lead salts, of a certain amount of petroselinic acid; alternatively, it must be borne in mind that there is some possibility that a linoleic acid corresponding with petroselinic acid may be present in fats of this type. If such an acid exists, the means at present employed in the isolation of petroselinic acid are insufficient to lead to its separation.

We may now proceed to tabulate the analytical data which we have obtained for the seven seed fats investigated.

Table III. *Fennel.*

Methyl esters of "solid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Estimated composition: Palmitate	Petroselinate	Unsaponifiable
S 1	6·5	129/144°	284·8	68·9	1·3	5·2	—
S 2	25·8	144/147	294·5	72·9	3·9	21·9	—
S 3	75·9	147/153	298·3	84·5	—	75·9	—
S 4	7·6	Residue	441·7 (345·7)	89·7	—	5·1	2·5
	115·8				5·2	108·1	2·5
				% as esters	4·4	93·4	2·2
				% as acids	4·4	93·3	2·3

Methyl esters of "liquid" acids.

					Palmitate	Oleate	Linoleate	Unsap.
L 1	6·5	80/142°	343·7 (279·5)	71·7	1·4	2·3	1·6	1·2
L 2	5·0	142/145	300·7 (283·7)	102·8	0·5	2·5	1·7	0·3
L 3	43·8	145/147	294·2	120·5	—	26·3	17·5	—
L 4	16·1	Residue	560·6 (345·9)	97·8	—	5·2	3·3	7·6
	71·4				1·9	36·3	24·1	9·1
				% as esters	2·6	50·9	33·7	12·8
				% as acids	2·6	50·6	33·5	13·3

Fraction	Fatty acids identified
S 1	Palmitic, M.P. 60–61° (*ca.* 20 % of fraction); 6 : 7-dihydroxystearic, M.P. 121°
S 3	6 : 7-Dihydroxystearic, M.P. 120–121°, and a trace of saturated acid
L 3	Dihydroxystearic mixture (?), M.P. 113–115°

Estimated composition of mixed fatty acids.

	"Solid" % 60·2	"Liquid" % 39·8	Total	Percentage (excluding unsaponifiable)
Palmitic	2·6	1·1	3·7	4
Petroselinic	56·2	—	56·2	60
Oleic	—	20·1	20·1	22
Linoleic	—	13·3	13·3	14
Unsaponifiable	1·4	5·3	6·7	—

Table IV. *Carrot.*

Methyl esters of "solid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Estimated composition: Palmitate	Petroselinate	Unsaponifiable
S 1	5·4	69/138°	291·5	73·2	0·8	4·6	—
S 2	33·1	138/149	293·9	81·6	1·6	31·5	—
S 3	12·4	149/152	295·4	84·6	0·1	12·3	—
S 4	34·1	152/153	296·4	86·0	—	34·1	—
S 5	11·5	Residue	326·1	80·0	—	10·4	1·1
	96·5				2·5	92·9	1·1
				% as esters	2·7	96·2	1·1
				% as acids	2·7	96·2	1·1

Methyl esters of "liquid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Palmitate	Oleate	Linoleate	Unsap.
L 1	7·1	118/149°	624·3 (282·0)	154·9 (93·1)	1·1	0·7	1·4	3·9
L 2	6·9	149/154	330·9 (298·4)	123·1 (103·6)	1·7	1·6	2·9	0·7
L 3	10·5	154/156	303·8	138·2	—	4·2	6·3	—
L 4	28·8	156/160	298·8	141·9	—	10·2	18·6	—
L 5	13·5	Residue	373·1	91·4	—	3·8	6·9	2·8
	66·8				2·8	20·5	36·1	7·4
				% as esters	4·1	30·8	54·1	11·0
				% as acids	4·1	30·6	53·8	11·5

Fraction	Fatty acids identified
S 1	Palmitic, M.P. 63·5° (*ca.* 20 % of fraction); 6 : 7-dihydroxystearic, M.P. 120–121°
S 4	6 : 7-Dihydroxystearic, M.P. 121–121·5°
L 4	Dihydroxystearic mixture (?), M.P. 113–115°

Estimated composition of mixed fatty acids.

	"Solid" % 57·1	"Liquid" % 42·9	Total	Percentage (excluding unsaponifiable)
Palmitic	1·5	1·8	3·3	4
Petroselinic	54·9	—	54·9	58
Oleic	—	13·1	13·1	14
Linoleic	—	23·1	23·1	24
Unsaponifiable	0·7	4·9	5·6	—

Table V. *Coriander.*

Methyl esters of "solid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Estimated composition: Palmitate	Petroselinate	Unsaponifiable
S 1	8·3	110/147°	278·8	54·0	3·1	5·2	—*
S 2	7·7	147/152	283·3	61·4	2·2	5·5	—
S 3	81·6	145/146	291·0	79·2	6·2	75·4	—
S 4	15·6	Residue	571·6	78·5	—	8·1	7·5
	113·2				11·5	94·2	7·5
				% as esters	10·2	83·2	6·6
				% as acids	10·1	83·0	6·9

Table V (*cont.*)

Methyl esters of "liquid" acids.

					Estimated composition			
					Palmitate	Oleate	Linoleate	Unsap.
L 1	6·2	70/140°	291·4	71·7	1·9	3·5	0·8	—
L 2	51·8	140/143	293·4	102·1	—	42·1	9·7	—
L 3	12·0	Residue	473·3	89·4	—	6·1	1·4	4·5
	70·0				1·9	51·7	11·9	4·5
				% as esters	2·6	73·9	17·1	6·4
				% as acids	2·6	73·7	17·0	6·7

Fraction	Fatty acids identified
S 1	Palmitic acid, M.P. 57–59° (*ca.* 15 % of fraction); 6 : 7-dihydroxystearic, M.P. 119–120°
S 2 and S 3	6 : 7-Dihydroxystearic, M.P. 120–121°, with traces of saturated acids
L 2	9 : 10-Dihydroxystearic, M.P. 128–129°, with some tetrahydroxystearic, M.P. 171–172°

Estimated composition of mixed fatty acids.

	"Solid" % 59·6	"Liquid" % 40·4	Total	Percentage (excluding unsaponifiable)
Palmitic	6·0	1·1	7·1	8
Petroselinic	49·5	—	49·5	53
Oleic	—	29·7	29·7	32
Linoleic	—	6·9	6·9	7
Unsaponifiable	4·1	2·7	6·8	—

Table VI. *Celery.*

Methyl esters of "solid" acids.

					Estimated composition			
No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Palmitate	Petroselinate	Linoleate	Unsaponifiable
S 1	4·2	85/143°	282·0	67·8	0·9	3·3	—	—
S 2	21·7	138/142	286·4	76·8	2·3	19·4	—	—
S 3	19·4	143/147	289·3	88·2	—	18·9	0·5	—
S 4	10·7	148/150	289·9	88·6	—	10·3	0·4	—
S 5	7·9	Residue	309·2	78·8	—	7·3	—	0·6
	63·9				3·2	59·2	0·9	0·6
				% as esters	4·9	92·7	1·4	1·0
				% as acids	4·9	92·7	1·4	1·0

Methyl esters of "liquid" acids.

					Palmitate	Oleate	Linoleate	Unsap.
L 1	4·2	86/155°	277·9	105·2	0·6	2·1	1·5	—
L 2	28·6	155/157	276·8	123·1	—	16·3	12·3	—
L 3	4·4	157/158	288·4	121·3	—	2·6	1·8	—
L 4	9·5	Residue	279·4	101·6	—	4·7	3·3	1·5
	46·7				0·6	25·7	18·9	1·5
				% as esters	1·3	55·0	40·4	3·3
				% as acids	1·3	54·9	40·3	3·5

Fraction	Fatty acids identified
S 2	Palmitic, M.P. 58–59°; 6 : 7-dihydroxystearic, M.P. 120–121°
S 3	6 : 7-Dihydroxystearic, M.P. 121–122°
L 2	Dihydroxystearic mixture (?), M.P. 113–115°, tetrahydroxsytearic, M.P. 170–171·5°

Table VI (*cont.*)

Estimated composition of mixed fatty acids.

	"Solid" % 54·0	"Liquid" % 46·0	Total	Percentage (excluding unsaponifiable)
Palmitic	2·6	0·6	3·2	3
Petroselinic	50·1	—	50·1	51
Oleic	—	25·3	25·3	26
Linoleic	0·7	18·5	19·2	20
Unsaponifiable	0·6	1·6	2·2	—

Table VII. *Parsnip.*

Methyl esters of "solid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Estimated composition: Palmitate	Petroselinate	Unsaponifiable
S 1	5·9	158/160°	281·1	79·9	0·4	5·5	—
S 2	18·5	160/161	285·0	85·5	—	18·5	—
S 3	4·4	Residue	350·0	79·4	—	4·1	0·3
	28·8				0·4	28·1	0·3
				% as esters	1·4	97·5	1·1
				% as acids	1·4	97·5	1·1

Methyl esters of "liquid" acids L 1

(from first crystallisation of lead salts).

					Oleate	Linoleate	Unsaponifiable
L 1/1	3·1	119/157°	291·4	130·6	1·5	1·6	—
L 1/2	4·6	157/159	294·5	127·6	2·4	2·2	—
L 1/3	4·8	Residue	300·5	111·2	2·2	2·0	0·6
	12·5				6·1	5·8	0·6
				% as esters	48·5	46·5	5·0
				% as acids	48·4	46·4	5·2

Methyl esters of "liquid" acids L 2

(from recrystallisation of lead salts).

					Palmitate	Oleate	Linoleate
L 2/1	4·0	108/154°	289·8	109·2	0·2	2·5	1·3
L 2/2	11·2	154/155	297·2	116·0	—	7·3	3·9
L 2/3	4·4	Residue	299·4	110·3	—	3·2	1·2
	19·6				0·2	13·0	6·4
				% as esters	1·2	66·0	32·8
				% as acids	1·2	66·0	32·8

Fraction	Fatty acids identified
S 2	6 : 7-Dihydroxystearic, M.P. 122°, with trace saturated acid
L 1/2	9 : 10-Dihydroxystearic, M.P. 129–130°
L 2/2	Dihydroxystearic mixture (?), M.P. 113–114°

Estimated composition of mixed fatty acids.

	"Solid" % 46·2	"Liquid" L 1 % 21·7	"Liquid" L 2 % 32·1	Total	Percentage (excluding unsaponifiable)
Palmitic	0·7	—	0·4	1·1	1
Petroselinic	45·0	—	—	45·0	46
Oleic	—	10·5	21·2	31·7	32
Linoleic	—	10·1	10·5	20·6	21
Unsaponifiable	0·5	1·1	—	1·6	—

Table VIII. *Chervil.*

Methyl esters of "solid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Estimated composition: Palmitate	Petroselinate	Linoleate	Unsaponifiable
S 1	3·4	90/141°	282·5	91·3	0·5	2·2	0·7	—
S 2	42·1	141/145	283·6	93·6	4·8	28·8	8·5	—
S 3	10·1	145/149	290·5	105·7	—	7·8	2·3	—
S 4	7·3	Residue	313·6	110·3	—	5·3	1·6	0·4
	62·9				5·3	44·1	13·1	0·4
				% as esters	8·4	70·1	20·8	0·7
				% as acids	8·4	70·1	20·8	0·7

Methyl esters of "liquid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Palmitate	Oleate	Linoleate	Unsap.
L 1	5·7	115/154°	285·9	153·5	0·6	0·1	5·0	—
L 2	68·3	154/155	298·5	172·3	-	0·4	67·9	—
L 3	3·8	155/157	297·7	170·5	—	0·1	3·7	—
L 4	10·4	Residue	292·4	135·7	—	0·2	8·1	2·1
	88·2				0·6	0·8	84·7	2·1
				% as esters	0·7	0·8	96·1	2·4
				% as acids	0·7	0·8	95·9	2·6

Fraction	Fatty acids identified
S 1	Palmitic, M.P. 57–59°; 6 : 7-dihydroxystearic, M.P. 120–121°
S 2	Palmitic, M.P. 60–61°; 6 : 7-dihydroxystearic, M.P. 121–122°
L 2	Dihydroxystearic mixture (?), M.P. 113–114°, tetrahydroxystearic, M.P. 169–170°, with traces of saturated acid (M.P. 50–52°) and water-soluble tetrahydroxystearic acid (M.P. 150°)

No linolenic acid was present (absence of insoluble hexabromides)

Estimated composition of mixed fatty acids.

	"Solid" % 57·5	"Liquid" % 42·5	Total	Percentage (excluding unsaponifiable)
Palmitic	4·8	0·3	5·1	5
Petroselinic	40·3	—	40·3	41
Oleic	—	0·3	0·3	0·5
Linoleic	12·0	40·8	52·8	53·5
Unsaponifiable	0·4	1·1	1·5	—

Table IX. *Caraway.*

Methyl esters of "solid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Estimated composition: Palmitate	Petroselinate	Unsaponifiable
S 1	12·5	123/130°	287·2	66·7	2·8	9·7	—
S 2	13·6	130/132	289·0	79·8	0·9	12·7	—
S 3	14·5	132/133	296·1	84·9	0·2	14·3	—
S 4	4·7	Residue	397·1 (355·6)	64·2 (63·9)	—	3·5	1·2
	45·3				3·9	40·2	1·2
				% as esters	8·6	88·7	2·7
				% as acids	8·6	88·6	2·8

Table IX (*cont.*)

Methyl esters of "liquid" acids.

					Estimated composition			
					Palmitate	Oleate	Linoleate	Unsap.
L 1	3·9	100/142°	310·7 (293·9)	114·6 (114·0)	0·3	1·9	1·5	0·2
L 2	18·9	142/144	295·9	112·0	—	13·2	5·7	—
L 3	49·3	144/146	298·2	123·7	—	27·9	21·4	—
L 4	23·7	146/148	296·0	129·4	—	11·8	11·9	—
L 5	13·8	148/149	295·9	127·3	—	7·2	6·6	—
L 6	15·6	Residue	469·3 (336·1)	122·1 (77·3)	—	5·1	4·7	5·8
	125·2				0·3	67·1	51·8	6·0
				% as esters	0·2	53·6	41·4	4·8
				% as acids	0·2	53·5	41·3	5·0

Fraction	Fatty acids identified
S 1	Palmitic, M.P. 61–62°; 6 : 7-dihydroxystearic, M.P. 121–122°
S 3	6 : 7-Dihydroxystearic, M.P. 120–121°
L 4	Mixture of dihydroxystearic acids

Estimated composition of mixed fatty acids.

	"Solid" % 28·7	"Liquid" % 71·3	Total	Percentage (excluding unsaponifiable)
Palmitic	2·5	0·1	2·6	3
Petroselinic	25·4	—	25·4	26
Oleic	—	38·2	38·2	40
Linoleic	—	29·4	29·4	31
Unsaponifiable	0·8	3·6	4·4	—

Discussion of results.

Inspection of the above tables shows that, whilst the quantitative interpretation of the results cannot reach the order of accuracy obtainable by the fractionation method in the absence of volatile resinous and unsaponifiable matter, the latter is concentrated for the most part in the methyl esters of those acids, the lead salts of which were soluble in alcohol. The analyses of the methyl esters derived from the insoluble lead salts are comparatively straightforward, and qualitative tests led to the ready identification of petroselinic acid, in the form of the characteristic 6 : 7-dihydroxystearic acid, and of palmitic acid. Consequently the figures quoted for the percentages of petroselinic acid are quite definite, and provide the necessary data for the main object of the investigation.

It is convenient to summarise the final results for the composition of the mixed fatty acids of each of the seven seed fats at this point.

Table X. *Percentage composition of fatty acids.*

	Palmitic	Petroselinic	Oleic	Linoleic
Fennel	4	60	22	14
Carrot	4	58	14	24
Coriander	8	53	32	7
Celery	3	51	26	20
Parsnip	1	46	32	21
Chervil	5	41	0·5	53·5
Caraway	3	26	40	31

Lino
Unsa

Quite evidently, petroselinic acid occurs in abundance in each of the seed fats now examined. The proportion in each case varies, however, somewhat widely, and whilst, on the one hand, the lowest figure (26 % in the case of caraway seed fat) is higher than that in *Heracleum* or *Angelica* (20 % [Hilditch and Jones, 1928]), the highest values now recorded (58 % in carrot seed fat and 60 % in fennel seed fat) are much lower than the corresponding value for parsley fat (76 % [Hilditch and Jones, 1927]). It is, of course, impossible to make any speculations as to the cause of the varying proportions of the acid in different genera, or even to suggest why Umbelliferae and some related orders of plants should elaborate an isomeric form in addition to the usual oleic acid. The variability in composition may, however, be contrasted with the case of the Palmae, in which genera so diverse as *Cocos nucifera*, *Elaeis guineensis* and *Attalea Cohune* contain not only the same mixture of fatty acids but (within 2 or 3 %) the same proportions of the predominating components, lauric, myristic and palmitic acids [Hilditch, 1928].

An attempt was made in the case of fennel, carrot and caraway seeds to determine whether the composition of the endosperm fat differed materially from that of the fatty oil present in the vittae. The dried seeds were ground up and then exposed to a current of air in such a way that the lighter fragments of the mericarp structure were displaced and the heavier particles collected. The results, however, were quite inconclusive, for on separating the fatty acids obtained from the fat in the concentrated heavy material by the lead salt method, the data observed were as follows:

Table XI.

	Fennel	Carrot	Caraway
Whole seed:			
% Fatty oil	13	16	9
"Solid" fatty acids: % of total acids	60	57	29
Iodine value	83 1	87·0	80·2
Separated seed:			
Percentage of whole seed	74	84	93
% Fatty oil	21	15	15
"Solid" fatty acids: % of total acids	63	47	19
Iodine value	81·2	82 5	71·9

So far as can be judged from this experiment, therefore, the variation in percentage of petroselinic acid does not seem to be connected with varying proportions of vittae fat and true seed fat in the different seeds.

The amount of palmitic acid, the only saturated acid present in these seed fats, is comparatively small and constant, the average figure being in the neighbourhood of 3–4 % of the total fatty acids.

Oleic and linoleic acids, which make up the remainder of the total fatty acids, are not present in constant relative proportions; but in the cases of the seed fats of fennel, carrot, celery, parsnip, and caraway the amount of oleic acid present is from 40–60 % of the combined oleic and linoleic acids, and in several cases the proportions are of much the same order.

The oleic and linoleic acid contents of chervil seed fat stand apart from the rest, since this portion of the chervil fatty acids was found to consist almost exclusively of linoleic acid; the tetrahydroxystearic acid of melting-point 169–170° was obtained in quantity from this acid, which therefore would appear to consist largely of the usual $\Delta^{9:10,\ 12:13}$-linoleic acid.

The low linoleic acid content of coriander seed fat may well be due to accidental circumstances, the seed in this case having deteriorated somewhat during storage; and therefore we do not attach too much significance to this particular figure.

Summary.

Seven further seed fats from cultivated species of the order Umbelliferae have been examined, and it has been found that $\Delta^{6\cdot 7}$-octadecenoic (petro-selinic) acid is present in quantity in all cases. The view that the presence of this acid is characteristic of Umbellate seed fats is thus strengthened.

The other components of the mixed fatty acids are palmitic acid in small quantity (1 8 %), together with $\Delta^{9\cdot 10}$-octadecenoic (ordinary oleic) acid and linoleic acid in somewhat varying proportions.

The fatty oils are accompanied in all cases by considerable amounts of unsaponifiable and resinous matter, which renders the quantitative investigation somewhat less certain than in other cases in which it has been applied.

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XLII. THE DISTRIBUTION OF REDUCING SUBSTANCES BETWEEN PLASMA AND CORPUSCLES; A COMPARISON OF VARIOUS BLOOD-SUGAR METHODS.

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THE distribution of "sugar" between plasma and corpuscles has been the subject of much discussion, and the results obtained by different workers have shown wide variation. We shall not attempt to survey the literature here; a summary is given in a paper by Wiechmann [1924]. In view of the work of de Wesselow [1919] it seemed probable that the variation might be, in part, explained by differences in the methods of blood-sugar estimation. De Wesselow compared MacLean's method with the Lewis-Benedict method, and found that whereas the discrepancies between the methods on plasma were small, MacLean's method always gave considerably lower figures on corpuscles.

In a preliminary experiment, on oxalated sheep's blood, we made a similar comparison between the methods of MacLean and of Folin and Wu. In addition to the direct determination, the corpuscle-sugar was calculated from the figures for plasma and whole blood, and the haematocrit reading for the corpuscle volume. The results were as follows (mg. per 100 cc.).

	Plasma	Whole blood	Corpuscle deposit	Corpuscles calculated
MacLean	48	30	0	0
Folin-Wu	51	50	35	48

These striking figures led us to undertake a fuller investigation of the distribution of reducing substances between plasma and corpuscles as determined by various methods, but for the main investigation we worked upon fresh human venous blood.

EXPERIMENTAL.

The subjects of our experiments were healthy adults, men and women, sometimes examined fasting, and sometimes after an ordinary meal or after a dose of glucose.

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[2] Working with grants from the Rosenberg-Stokvis-Bischoffsheim funds.

"Sugar" was determined directly in whole blood, plasma, and corpuscles. In addition, the corpuscle-sugar was calculated from the figures found for whole blood and plasma and the haematocrit reading for the corpuscle volume. As the figures determined directly on the corpuscle deposit were open to objections (they might have been too high as a result of insufficiently close packing of corpuscles, or too low owing to glycolysis during the time of spinning) we have tabulated only the calculated figures for corpuscles.

In the majority of experiments the procedure was as follows. A sample of blood taken by vein puncture was run into a paraffin-coated tube, and a second sample taken immediately after into a tube containing potassium oxalate. The oxalated blood was used for determinations of sugar in whole blood. The sample in the paraffin-coated tube was centrifuged at once for 2 4 minutes at 3000 r.p.m., and the plasma was pipetted off and oxalated. The corpuscle deposit was oxalated and centrifuged for a further 10 minutes. There was very rarely any clotting in the paraffin-lined tube. This procedure is indicated in the tables as "paraffin-tube technique." In oxalating the samples of whole blood, and of plasma and corpuscles after separation, care was taken to avoid an excess of oxalate—the amount present was approximately 0·15 to 0·3 %.

In a few experiments the whole sample of blood was defibrinated, and no anticoagulant used at any stage; in a few others the whole sample was oxalated.

We were able to complete the measurement of our samples into the appropriate diluting fluids in less than half an hour from the time of puncture, even when many methods were used. Thus there is little risk of error due to glycolysis.

The methods we have used are:

(1) MacLean's method [1919];

(2) the Folin-Wu method [1920];

(3) the Shaffer-Hartmann method as modified by Somogyi [1926];

(4) the method of Hagedorn and Jensen as originally described, using zinc hydroxide filtrates [1923];

(5) the method of Hagedorn and Jensen applied to tungstic filtrates [Hiller, Linder and Van Slyke, 1925; Hawk and Bergeim, 1926];

(6) the modification of the Folin-Wu method recently introduced by Benedict [1928] in which the sensitiveness of the copper reagent is diminished by addition of alanine, sodium nitrate and $NaHSO_3$. This method is indicated in the tables as the Benedict (1928).

A further note on the preparation of the tungstic filtrates is necessary. The amounts of 10 % sodium tungstate and 2/3 N sulphuric acid were adjusted according to the different protein content of plasma, whole blood, and corpuscles. For 1 volume plasma, ½ volume of each of the reagents was used; for 1 volume whole blood, 1 volume of each; and for 1 volume corpuscles, 1½ volumes of each; the total being always made up to 10 volumes. These

tungstic filtrates were used for four different methods—Folin-Wu, Shaffer-Hartmann, Hagedorn and Jensen, and Benedict (1928).

In using the colorimetric methods, a standard of approximately the same strength as the blood filtrate was always used.

All determinations were made in duplicate.

We have checked our technique in all the methods by experiments with glucose solutions and glucose added to blood.

Our results are given in Tables I and II.

Table I. *Comparisons between the MacLean and Folin-Wu methods.*

Date	Subject	Remarks		Reducing substance as glucose mg. per 100 cc. Plasma	Whole blood	Corpuscles	Ratio Plasma "sugar" / Corpuscle "sugar"
24. vii. 28	J.G.	1¾ hrs. after lunch. Paraffin tube technique	MacLean	93	78	57	1·63
			Folin-Wu	102	99	95	1·07
27. vii. 28	J.G.	¾ hr. after lunch. P. tube technique	MacLean	94	81	71	1·32
			Folin-Wu	112	110	107	1·04
4. ix. 28	J.G.	2 hrs. after lunch. P. tube technique	MacLean	102	89	71	1·43
			Folin-Wu	106	104	101	1·04
5. ix. 28	J.G.	P. tube technique. Fasting	MacLean	94	84	71	1·32
			Folin-Wu	106	100	92	1·05
		½ hr. after 50 g. glucose	MacLean	160	140	114	1·40
			Folin-Wu	167	154	137	1·22
		1 hr. after glucose	MacLean	161	136	104	1·55
			Folin-Wu	166	148	125	1·32
		2 hrs. after glucose	MacLean	92	84	74	1·24
			Folin-Wu	88	93	102	0·86
7. ix. 28	K.	2¾ hrs. after lunch. P. tube technique	MacLean	92	80	61	1·51
			Folin-Wu	99	97	94	1·05
23. vii. 28	F.K.H.	1 hr. after lunch. P. tube technique	MacLean	117	109	95	1·23
			Folin-Wu	121	120	118	1·03

Summary of results.

The methods first compared were those of MacLean and of Folin and Wu. The discrepancies between the two methods will be considered first, and the other methods then compared with these.

MacLean's method compared with the method of Folin and Wu.

There are sixteen comparisons between these two methods. If discrepancies in which the Folin-Wu figure is higher than the MacLean figure are taken as positive, and the reverse discrepancies as negative, we find:

on plasma,	the range of discrepancy is	− 4 to + 18,	average	+ 8 mg. per 100 cc.
„ whole blood,	„ „	+ 9 to + 36,	„	+ 19 „
„ corpuscles,	„ „	+ 19 to + 49,	„	+ 31 „

The range of variation is wide, but in every experiment the same effect is shown to a greater or less degree. The discrepancy between the two methods

Table II. *Distribution of reducing substances between plasma and corpuscles as determined by various blood-sugar methods.*

Date	Subject	Conditions	Methods	Reducing substance as glucose mg. per 100 cc. Plasma	Whole blood	Corpuscles	Ratio Plasma "sugar" / Corpuscle "sugar"
17. x. 28	R.J.B.	1¼ hrs. after lunch	MacLean	120	113	105	1·14
		P. tube	Hagedorn (zinc)	—	110	—	—
		technique	,, (tungstic)	124	140	162	0·76
			Folin-Wu	131	128	124	1·06
			Shaffer-Hartmann	136	135	135	1·01
21. x. 28	F.K.H.	2 hrs. after lunch.	MacLean	104	83	50	2·08
		Oxalated	Hagedorn (zinc)	99	85	63	1·57
		blood	,, (tungstic)	86	103	130	0·66
			Folin-Wu	109	109	109	1·00
			Shaffer-Hartmann	105	105	105	1·00
24. x. 28	P.	Immediately after tea.	Hagedorn (zinc)	148	133	112	1·23
		P. tube	,, (tungstic)	163	170	180	0·91
		technique	Folin-Wu	160	158	155	1·03
			Shaffer-Hartmann	158	159	161	0·98
			Benedict (1928)	162	150	132	1·23
26. x. 28	K.	2 hrs. after lunch.	Hagedorn (zinc)	77	71	62	1·24
		P. tube	,, (tungstic)	89	101	118	0·75
		technique	Folin-Wu	89	87	85	1·05
			Shaffer-Hartmann	84	83	84	1·00
			Benedict (1928)	90	81	68	1·32
29. x. 28	G.A.H.	1½ hrs. after lunch.	MacLean	95	81	62	1·53
		P. tube	Hagedorn (zinc)	110	94	72	1·53
		technique	,, (tungstic)	106	110	116	0·92
			Folin-Wu	106	101	94	1·11
			Shaffer-Hartmann	110	102	91	1·21
			Benedict (1928)	110	96	77	1·43
1. xi. 28	B.	After lunch.	Hagedorn (zinc)	108	106	103	1·05
		P. tube	,, (tungstic)	114	131	153	0·75
		technique	Folin-Wu	120	115	109	1·10
			Shaffer-Hartmann	115	115	115	1·00
			Benedict (1928)	118	105	86	1·25
5. xi. 28	K.M.H.	3 hrs. after lunch.	MacLean	137	127	113	1·21
		P. tube technique	Hagedorn (zinc)	142	124	98	1·45
7. xi. 28	G.	1½ hrs. after lunch.	MacLean	112	102	90	1·24
		P. tube	Hagedorn (zinc)	119	107	93	1·28
		technique	,, (tungstic)	141	134	126	1·12
			Folin-Wu	123	119	114	1·08
			Shaffer-Hartmann	120	112	103	1·17
13. xi. 28	F.K.H.	After lunch.	MacLean	99	81	51	1·94
		P. tube	Hagedorn (zinc)	95	80	55	1·73
		technique	,, (tungstic)	100	110	127	0·78
			Folin-Wu	102	99	94	1·06
			Shaffer-Hartmann	97	97	97	1·00
			Benedict (1928)	105	89	62	1·69
14. xi. 28	R.J.B.	3 hrs. after lunch.	MacLean	95	85	69	1·38
		Oxalated	Hagedorn (zinc)	98	85	66	1·52
		blood	,, (tungstic)	106	124	156	0·68
			Folin-Wu	109	111	114	0·96
			Shaffer-Hartmann	102	109	120	0·85
			Benedict (1928)	116	113	108	1·07
15. xi. 28	L.R.W.P.	3 hrs. after lunch.	MacLean	105	94	80	1·38
		Defibrinated	Hagedorn (zinc)	114	104	92	1·24
		blood	,, (tungstic)	119	132	149	0·80
			Folin-Wu	118	120	122	0·97
			Shaffer-Hartmann	116	114	112	1·04
			Benedict (1928)	112	107	101	1·11
7. i. 29	F.K.H.	2 hrs after lunch.	MacLean	82	62	33	2·48
		Defibrinated blood	Shaffer-Hartmann	86	86	86	1·00

is always greater on whole blood than on plasma, and greatest on the corpuscle figures. This was confirmed by the direct determinations on corpuscle deposits, which have not been tabulated.

The Shaffer-Hartmann method as modified by Somogyi.

This method gives figures agreeing well with the Folin-Wu figures, and, like them, showing a small discrepancy with the MacLean results on plasma, and a marked difference on corpuscles. Discrepancies in which the Shaffer-Hartmann figures are higher have been taken as positive, and the reverse discrepancies as negative, in each case.

There are ten comparisons with the Folin-Wu method, and the discrepancies between Shaffer-Hartmann figures and Folin-Wu figures are:

on plasma:	− 7 to + 5,	average − 3	mg. per 100 cc.
„ whole blood:	− 6 to + 7,	„ 0	„
„ corpuscles:	− 9 to + 11,	„ 0	„

There are eight comparisons with MacLean's method, and the discrepancies are:

on plasma:	2 to + 15,	average + 7	mg. per 100 cc.
„ whole blood:	+ 10 to + 24,	„ + 20	„
„ corpuscles:	+ 13 to + 55,	„ + 39	„

The method of Hagedorn and Jensen.

The original method, applied to zinc hydroxide filtrates, gives figures which agree fairly well with MacLean's method, and shows definitely lower figures for corpuscles than for plasma. When the ferricyanide reduction method is applied to tungstic acid filtrates, the results indicate a very high corpuscle-sugar - the highest given by any method. This is the only method which indicates a definitely higher sugar value for corpuscles than for plasma. We will summarise these effects by comparing the original Hagedorn and Jensen method with MacLean's method, the Hagedorn and Jensen method (tungstic filtrates) with the Folin-Wu method, and thirdly, comparing the zinc filtrates with the tungstic filtrates.

The original Hagedorn and Jensen method compared with MacLean's method. There are seven comparisons. If the discrepancies in which the Hagedorn and Jensen method gives the higher figure are taken as positive, the results are:

on plasma,	the range of discrepancy is	− 5 to + 15,	average + 4	mg. per 100 cc.
„ whole blood,	„ „	− 3 to + 12,	„ + 4	„
„ corpuscles,	„ „	− 14 to + 13,	„ + 4	„

In most of the determinations the figures given by the two methods agree closely, and, where there is a definite difference, the method of Hagedorn and Jensen gives the higher figure, and the difference is about the same on plasma and corpuscles.

The Hagedorn and Jensen method, applied to tungstic filtrates, compared with the Folin-Wu method. There are ten comparisons, and, taking discrepancies in which the Hagedorn and Jensen figures are higher as positive, we have:

on plasma,	the range of discrepancy is	− 23 to + 18,	average	+ 2 mg. per 100 cc.
,, whole blood,	,, ,,	− 6 to + 16,	,,	+ 11 ,,
,, corpuscles,	,, ,,	+ 12 to + 44,	,,	+ 30 ,,

The wide range of discrepancy on plasma is chiefly due to two extreme experiments. In the remaining eight experiments the range is − 7 to + 1 (average − 2).

The method of Hagedorn and Jensen: zinc filtrates and tungstic filtrates. There are ten comparisons. Discrepancies in which the tungstic filtrates give the higher figures are taken as positive.

on plasma,	the range of discrepancy is	− 13 to + 22,	average	+ 6 mg. per 100 cc.
,, whole blood,	,, ,,	+ 16 to + 39,	,,	+ 27 ,,
,, corpuscles,	,, ,,	+ 33 to + 90,	,,	+ 56 ,,

Benedict's modification of the Folin-Wu method.

Compared with the original Folin-Wu method Benedict's modification gives slightly lower figures on whole blood. Taking discrepancies in which the Folin-Wu method gives higher figures as positive, we have:

on plasma,	the range of discrepancy is	− 7 to + 6,	average	− 1 mg. per 100 cc.
,, whole blood,	,, ,,	+ 2 to + 13,	,,	+ 8 ,,
,, corpuscles,	,, ,,	+ 6 to + 32,	,,	+ 20 ,,

These figures are based on seven comparisons.

The Benedict (1928) method compared with the original Hagedorn and Jensen method. We take this comparison rather than the comparison with MacLean's method, because we have seven experiments in which the Benedict and Hagedorn and Jensen methods are compared, and only four in which there are both MacLean figures and Benedict figures.

If we take discrepancies in which the Benedict figures are higher than the Hagedorn and Jensen figures as positive, we have:

on plasma,	the range of discrepancy is	− 2 to + 14,	average	+ 9 mg. per 100 cc.
,, whole blood,	,, ,,	− 1 to + 28,	,,	+ 10 ,,
,, corpuscles,	,, ,,	− 17 to + 42,	,,	+ 10 ,,

The ratio of plasma-"sugar" to corpuscle-"sugar."

It has already been explained that, in the majority of our experiments, the plasma was obtained from a sample of blood to which no anti-coagulant was added until the plasma had been separated. Under these conditions the true ratio of "sugar" in plasma and corpuscles should be obtained. The average ratios of plasma-"sugar" to corpuscle-"sugar" by the various methods (excluding the few experiments on oxalated blood) are as follows:

MacLean	1·48 (average of 16 determinations)
Hagedorn-Jensen (zinc filtrates)	1·34 (,, 8 ,,)
Hagedorn-Jensen (tungstic filtrates)	0·85 (,, 8 ,,)
Folin-Wu	1·07 (,, 17 ,,)
Shaffer-Hartmann	1·05 (,, 9 ,,)
Benedict (1928)	1·34 (,, 6 ,,

The difference between the methods of MacLean and Hagedorn and Jensen (zinc filtrates) may be exaggerated here, because there are several experiments in which only one of these methods was used. If we take the shorter series of experiments in which both methods were applied to the same blood, the average ratios are much closer together (viz. 1·46 and 1·45). There is considerable variation of the ratio in all the three methods which give the higher ratios, and the number of experiments is not large enough to give strictly comparable averages. A consideration of individual experiments brings out the same relations as are shown by the average figures.

It is noteworthy that in the sugar-tolerance curve (5. ix. 28) the ratios by both MacLean's method and the Folin-Wu method rose when the total sugar was high, and fell when the total sugar was low, whereas the discrepancies between the methods remained fairly steady.

To sum up: we have two methods which agree fairly well and indicate a higher sugar value in plasma than in corpuscles, namely MacLean's method and the original Hagedorn and Jensen method. The Benedict (1928) method[1] gives a similar distribution ratio, but this ratio is derived from slightly higher figures for both the plasma and corpuscles. We have two methods which agree very well and indicate an approximately even distribution of "sugar" between plasma and corpuscles—the Folin-Wu and Shaffer-Hartmann methods. Finally, the Hagedorn and Jensen method applied to tungstic filtrates is alone in indicating a higher sugar figure for corpuscles than for plasma, thus differing very greatly from the same method of estimation applied to zinc hydroxide filtrates.

Possible effects due to technique.

Before discussing the significance of these results we must consider two factors inherent in the technique, which might unequally affect the determinations on plasma, whole blood, and corpuscles.

The first is the possible error introduced by taking an aliquot part of a blood filtrate. The original method of Hagedorn and Jensen is free from this objection, because the protein precipitate is washed and the whole filtrate with washings is used. In the MacLean method of protein precipitation the blood is diluted 1 in 125, so that the volume of the precipitate is negligible. In the tungstic precipitation the conditions are different. The dilution of the blood is 1 in 10, and there is an apparently voluminous protein precipitate which is greater in whole blood than in plasma, and greater still when corpuscles are used. We have observed that there is no volume change on mixing the reagents: the total volume of the mixture, including the precipitate, is 10 cc. for 1 cc. of blood. If the volume of the precipitate is appreciable, the filtrate must be concentrated, and since, when taking an aliquot part, we

[1] Rockwood [1926] has made comparisons between the Folin-Wu method and Benedict's earlier modification of it (not the method we have used). He found that the differences between the original Folin-Wu and the modification were most pronounced in the corpuscle figures.

assume that the fluid volume is still 10 cc., our figures will be too high, the error being greatest when the protein precipitate is greatest. We tested this experimentally as follows. The usual mixtures of plasma, whole blood, or corpuscles with the sodium tungstate and sulphuric acid were made in graduated centrifuge tubes; the protein was centrifuged down and the supernatant liquid removed, and the tubes were dried to constant weight. A known volume of absolute alcohol was pipetted into each tube and the level noted. In this way the solid volume was estimated by displacement; for plasma we found it 0·5 % of the total volume of precipitation mixture, for whole blood 2 %, and for corpuscles 2·5 %. These figures are only approximate, but show that the effect is negligible and that no appreciable error is introduced by taking aliquot parts of the filtrate.

Secondly, in the tungstic precipitation and the MacLean precipitation the reagents are acid, and some hydrogen ions are taken up by the protein. Since there is less protein in the plasma and more in the corpuscles, the acidity of the filtrates from plasma is greater than that of filtrates from whole blood. In our tungstic filtrates from plasma the p_H was approximately 4·8, from the whole blood 5·1, and from corpuscles 5·3. There was thus little difference, and we found that adjustment of the filtrates to approximately p_H 6·0 had no effect on the figures obtained. The copper reduction methods are sensitive to changes in p_H, as has been emphasised by Folin and Svedberg [1926] and Somogyi [1926], but in our filtrates the difference was too small to introduce any error. Similarly, in the MacLean method, the differences in acidity of filtrates are too small to affect the results. In the zinc hydroxide filtrates used for the Hagedorn and Jensen method, there is no appreciable difference in p_H between filtrates from plasma, whole blood, and corpuscles; all three are approximately of p_H 6·6.

A non-glucose reducing substance.

We know that all the methods estimate glucose quantitatively, whether in pure solution or added to blood. It is therefore unlikely that the methods which give low figures on corpuscles are failing to estimate glucose, and we are led to postulate the presence of some non-glucose reducing substance, predominantly present in corpuscles, which may account for the discrepancies between methods. The methods giving lower, and therefore probably truer, glucose figures are the methods of MacLean and Hagedorn and Jensen (zinc filtrates). These methods are absolutely different in principle, and yet give fair agreement. But when we apply the ferricyanide reduction to tungstic filtrates we obtain the highest figures of any for corpuscle-sugar. The interfering substances must be present in tungstic filtrates, and either absent from the iron filtrates and zinc filtrates, or present in them in smaller amount. Of the four methods applied to tungstic filtrates, three give high corpuscle figures—Hagedorn and Jensen, Shaffer-Hartmann, and Folin-Wu—and the tungstic precipitation is the only feature common to the three methods. The

Benedict (1928) method is the only method applied to tungstic filtrates which gives a distribution ratio similar to that given by MacLean's method, and the copper reagent used in the Benedict (1928) method is designed to be relatively insensitive, and therefore more selective than the copper reagent of the Folin-Wu method. Benedict claims that his modification gives true sugar figures, but it seems probable that the figures given by his method, though lower than the Folin-Wu figures, are slightly above the true sugar value, since we find that the methods of MacLean and Hagedorn and Jensen (zinc filtrates) give lower figures still.

We have further evidence of the important part played by the method of protein precipitation. We have applied MacLean's method to tungstic filtrates as follows. 2 cc. of a 1 in 10 tungstic filtrate are diluted with 18 cc. of the acid sodium sulphate solution used in MacLean's method: 2 cc. of the copper solution are added, and the estimation carried out in the usual way. (The filtrate taken corresponds to 0·2 cc. blood, *i.e.* 5/1 of the amount in the ordinary MacLean method.) This modification of the method quantitatively determines glucose added to blood. In Table III are given comparisons between the ordinary MacLean method, the MacLean method applied to tungstic filtrates, and the Folin-Wu or Shaffer-Hartmann methods. MacLean's method of estimation gives higher figures on tungstic than on iron filtrates, and the MacLean estimation applied to tungstic filtrates usually agrees with the Folin-Wu or Shaffer-Hartmann method. The distribution ratio given by the MacLean method applied to tungstic filtrates is close to unity, and differs greatly from the ratio given by the ordinary MacLean method.

Table III.

	Methods		Reducing substance as glucose, mg. per 100 cc. Plasma	Whole blood	Corpuscles (directly determined)
I	MacLean	Iron filtrate	—	75	—
	,,	Tungstic filtrate	—	83	—
	Folin-Wu	,,	—	102	—
II	MacLean	Iron filtrate	120	113	—
	,,	Tungstic filtrate	124	123	—
	Folin-Wu	,,	131	128	—
III	MacLean	Iron filtrate	-	87	—
	,,	Tungstic filtrate	—	94	—
	Folin-Wu	,,	—	100	—
IV	MacLean	Iron filtrate	101	84	78
	,,	Tungstic filtrate	120	118	109
	Shaffer-Hartmann	,,	—	116	115
V	MacLean	Iron filtrate	82	62	—
	,,	Tungstic filtrate	91	88	88
	Shaffer-Hartmann	,,	86	86	86

We are thus led to the hypothesis that there is some non-glucose reducing substance present in corpuscles. The various methods applied to tungstic filtrates vary in their sensitiveness to this unknown substance, the ferri-

cyanide reduction being the most sensitive, and the Benedict (1928) copper reduction the least.

Direct evidence of a non-glucose reducing substance in corpuscles. In addition to the inference drawn from our comparison of methods, we have direct evidence of a reducing substance other than glucose in tungstic filtrates of whole blood or corpuscle deposits.

If a tungstic filtrate from whole blood is added to the alkaline copper reagent used in the Folin-Wu estimation, and the phosphomolybdate reagent is added at once, in the cold, a definite blue colour develops. This reaction is not given by plasma filtrates, and is given very strongly by corpuscle filtrates. The reaction does not occur if the blood filtrate and phosphomolybdate are mixed in the absence of the copper solution; it is a reduction of the copper reagent, not a direct reduction of the phosphomolybdate reagent.

Colloidal ferric hydroxide filtrates from blood do not give the reaction with the Folin-Wu copper solution, neither do zinc hydroxide filtrates. For the purpose of this test the iron or zinc filtrates were of the same concentration as the tungstic filtrates—10 cc. filtrate corresponding to 1 cc. blood. We have therefore direct evidence of a reducing substance in corpuscles, which passes into tungstic filtrates and not into iron or zinc filtrates.

It is possible to remove glucose from blood by a short incubation with a large excess of yeast, and to precipitate the mixture with tungstic acid [Somogyi, 1927]. Such filtrates from fermented blood still give the reaction with the Folin-Wu copper solution in the cold.

The probable nature of the unknown substance. The idea that non-glucose reducing substances in blood may affect blood-sugar estimations is not new; it is known that some of the methods give a residual reduction after fermentation with yeast. The earlier work on this residual reduction was unsatisfactory, owing to technical difficulties, but these were overcome by the discovery of Hiller, Linder and Van Slyke [1925], that a very short incubation, with excess of yeast, was sufficient to remove glucose. Somogyi, using this principle, has determined the non-fermentable reducing substances in whole blood, plasma and corpuscles, by his modification of the Shaffer-Hartmann method. In his first paper [1927] he gave the figure for whole blood as 27 mg. per 100 cc., but his later technique [1928] gave figures 3 or 4 mg. lower; on this basis we may take the figure for plasma as 8, for whole blood 23, and for corpuscles 40 mg. per 100 cc. Now we have found that MacLean's method gives figures lower than the Shaffer-Hartmann method as modified by Somogyi, and that the discrepancy is for plasma 7, for whole blood 20, and for corpuscles 39 mg. per 100 cc. These are average figures. The agreement between these discrepancies and Somogyi's figures for non-glucose reducing substances is striking, and naturally leads to the suggestion that MacLean's method gives figures very close to the true sugar value.

We must now consider some substances which might be responsible for the residual reduction after fermentation with yeast, and for the discrepancies between methods.

Uric acid, creatine and creatinine may be dismissed, for Hiller, Linder and Van Slyke [1925] have shown that their reduction effect is too small to be of importance, in view of the small amounts present in blood.

Ergothioneine is a reducing substance, present only in corpuscles, and it is capable of affecting blood-sugar methods [Sjollema, 1927]. Hunter [1928] has estimated the amount present in normal human blood, and finds 2–10 mg. per 100 cc. corpuscles, *i.e.* a maximum of 4 or 5 mg. per 100 cc. whole blood. We have made a few determinations of the effect of ergothioneine solutions upon the various blood-sugar methods, and find that 100 mg. ergothioneine is equivalent to 10–60 mg. glucose, according to the method used. In view of the very small amount of ergothioneine in human blood, the effect is negligible.

Glutathione is known to be present in corpuscles, mainly in the reduced form [Hunter and Eagles, 1927; Holden, 1925; Thomson and Voegtlin, 1926; Uyei, 1926]. It is capable of reducing ferricyanide and the Folin-Wu reagents [Sjollema, 1927]. We have found that solutions of reduced glutathione reduce the Folin-Wu copper reagent in the cold; glutathione is therefore probably responsible for this reaction as given by tungstic filtrates of corpuscles.

It is well known that tungstic filtrates from corpuscles give a positive nitroprusside reaction, whereas tungstic filtrates from plasma do not. On the basis of the intensity of the nitroprusside reaction, Hunter and Eagles estimate the glutathione in human blood at 100–120 mg. per 100 cc. corpuscles, *i.e.* 40–50 mg. per 100 cc. blood.

The subject of the quantitative effect of glutathione in the various blood-sugar methods, and, in particular, its behaviour in the various methods of protein precipitation, is still under investigation, and will form the subject of a later paper. It may be said at present that concentrations of reduced glutathione, of the order of 45 mg. per 100 cc., give a significant reduction of all the blood-sugar reagents except that of Benedict (1928).

Significance for general analytical work.

We have shown that differences of the order of 30 mg. per 100 cc. are commonly found, when different methods are applied to the same sample of whole blood. Differences of this order are of importance in clinical work, and it is necessary to interpret results according to the method used. Also our findings give an explanation of some of the varying results obtained by different workers for the distribution of "sugar" between plasma and corpuscles. Further, our results suggest that the choice of method may be of great importance in the determination of "sugar" in tissue extracts or other material in which glutathione and other reducing substances are likely to be present, particularly if the relative amount of glucose is small and concentrated extracts are used (*e.g.* in glycolysis experiments).

Summary.

1. The various methods of blood-sugar estimation give widely different results for the distribution of "sugar" between plasma and corpuscles. The average discrepancies between methods are not great for estimations on plasma, but are considerable for estimations on corpuscles.

2. The methods of MacLean and Hagedorn and Jensen (original) agree fairly well, and give a higher sugar value in plasma than in corpuscles.

3. The methods of Folin-Wu and Shaffer-Hartmann (Somogyi's modification) agree very well, and indicate approximately even distribution of reducing substances between plasma and corpuscles.

4. In comparing the method of MacLean or Hagedorn and Jensen (original), with the method of Folin-Wu or Shaffer-Hartmann, we find that the first pair of methods gives slightly lower figures than the second pair of methods on plasma, and considerably lower figures on corpuscles.

5. Benedict's latest modification of the Folin-Wu method gives ratios of plasma-"sugar" to corpuscle-"sugar" similar to those given by the method of MacLean or the original Hagedorn and Jensen method, but the figures given by the Benedict (1928) method are slightly higher on both plasma and corpuscles.

6. The method of Hagedorn and Jensen applied to tungstic filtrates gives very high corpuscle figures, and is the only method which shows more "sugar" in corpuscles than in plasma. Zinc hydroxide filtrates from whole blood or corpuscles give very much lower figures than tungstic filtrates when estimated by the ferricyanide method.

7. MacLean's method may be applied to tungstic filtrates and the results for corpuscles are much higher, by this modification, than by the original method.

8. We suggest that the discrepancies between blood-sugar methods are due to the presence of a non-glucose reducing substance in corpuscles, which is present in tungstic filtrates, and is either absent from iron and zinc filtrates, or present in them in less amount. We have direct evidence of the presence of such a substance, because tungstic filtrates of whole blood or corpuscles produce a reduction of the Folin-Wu copper reagent in the cold, whereas filtrates from plasma give no such reaction. The reaction is not given by iron filtrates or zinc filtrates.

9. It is suggested that the substances responsible for the discrepancies between methods may also be responsible for the residual reduction after fermentation of blood with yeast.

10. Uric acid, creatine, creatinine, and ergothioneine are dismissed as having no appreciable effect on blood-sugar methods, in the small amounts in which these substances occur in human blood.

11. Glutathione is suggested as having an important effect on blood-sugar methods.

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XLIII. MEMBRANE EQUILIBRIA AND SELECTIVE ABSORPTION.

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Two general theories have been advanced to account for the unequal distribution of inorganic electrolytes on either side of a cell membrane.

According to one theory, the unequal distribution is due to the existence of a cell membrane, which not only prevents the passage of the colloid constituents of the cell, but also has the property of presenting varying resistance to the passage of different inorganic ions. According to the other theory, the cell membrane is capable of preventing the passage of the colloid constituents of the cell, but the unequal distribution of inorganic salts is due to the formation within the cell of relatively undissociated and non-diffusible compounds of the various ions with the cell constituents.

Direct experiments designed to test the former theory are beset with considerable difficulties, since the experiments have to be carried out on living tissues, and any considerable alterations in the surrounding medium are liable to alter the properties of the membrane. On the other hand, many artificial membranes exist which may be used in testing the latter theory, since the only properties which the membrane must have are those of preventing the passage of colloids and of allowing the free passage of inorganic salts.

In previous work [Wright, 1926] an attempt was made to ascertain how far the secretion of high concentrations of calcium in milk from the relatively low concentrations of this element in the blood-plasma could be accounted for on the basis of an unequal distribution caused by the presence in the milk cells of non-diffusible and relatively undissociated protein molecules. For this purpose, solutions of sodium caseinogenate were separated from solutions of calcium salts by a membrane permeable to inorganic ions but impermeable to the caseinogen molecules or ions. It was found that under these conditions the calcium tended to accumulate in high concentration on the protein side of the membrane, the distribution ratios[1] for the calcium varying from 2·2 to as high as 12·8. No attempt was made in the preliminary paper to examine in detail the physical chemistry of the systems under investigation.

[1] Throughout this paper, the term "distribution ratio" indicates, for cations, the ratio of the concentration on the protein side of the membrane to that on the non-protein side, and for anions, the ratio of the concentration on the non-protein side to that on the protein side.

The results appeared, however, to have such important applications in the more general study of the unequal distribution of salts in living tissues, that it was decided to carry out further, and more detailed, experiments. The results of these later experiments, which are described in this paper, show conclusively that (in the systems studied) the observed inequalities in the distribution ratios of the salts can be entirely explained if two factors—the degree of dissociation of the protein salts and the establishment of a Donnan equilibrium—are taken into account.

Technique.

100 cc. of each of the two solutions under investigation were placed on either side of a dialysing vessel, the solutions were allowed to come into equilibrium by dialysing for at least 24 hours, and the contents were then removed and analysed.

The caseinogen used in the experiments was prepared by the method detailed by Van Slyke [1923] which gives a product of remarkably low ash content. The ash in the samples used was less than 0·1 %.

The dialysing vessels were of the type devised by Wright and Rule [1927], cellophane (0·0017 cm. thick) being employed as the membrane. This membrane is impermeable to caseinogen but freely permeable to inorganic salts.

With regard to analysis, calcium was estimated by McCrudden's [1909] method, and chloride by a macro-method based on that of Van Slyke [1924]. Sodium was determined throughout by difference[1]. The hydrogen ion concentration of the solutions was not rigidly controlled, but the solutions were in all cases kept between p_H 6·7 and 7·0. It will be seen from the results that the small variations had no significant effect on the equilibria obtained. Toluene was added in order to prevent bacterial decomposition of the protein.

Results.

Table I shows the results obtained with simple systems containing Na caseinogenate and NaCl. It will be seen that, at equilibrium, the distribution ratios of the sodium are in every case greater than the corresponding ratios of the chloride.

If Donnan's theory holds for such systems, this inequality in the distribution ratios can only be accounted for on the assumption that the Na caseinogenate is not completely dissociated. By calculation from the experimental data, it is found that a percentage dissociation of the caseinogenate of between 62 and 77 % (with a mean value of 68 %) is necessary in order to make the ionic ratios equal. Such a value agrees closely with that obtained from conductivity data by Pauli [1922].

[1] *I.e.* total anions – total calcium, assuming that 5 % caseinogen acts as a 0·025 *N* anion. All calculations in this paper are based on the analytical data, and not on the quantities of electrolytes noted as initially present. The latter are approximate values only.

Table I. *Equilibria in systems containing sodium caseinogenate and sodium chloride.*

	Approximate initial concentrations			Concentrations at equilibrium				Ratios at equilibrium		Percentage dissociation of caseinogenate
	Side I		Side II	Sodium		Chloride				
Exp. No.	Caseinogen %	NaOH N	NaCl N	I N	II N	I N	II N	$\frac{Na_I}{Na_{II}}$	$\frac{Cl_{II}}{Cl_I}$	
1	5·0	0·0250	0·1000	0·0688	0·0526	0·0438	0·0526	1·31	1·20	77
2	5·0	0·0250	0·0500	0·0443	0·0269	0·0193	0·0269	1·65	1·39	67
3	5·0	0·0250	0·0250	0·0337	0·0145	0·0087	0·0145	2·32	1·67	62
4	5·0	0·0250	0·0125	0·0288	0·0088	0·0038	0·0088	3·28	2·31	66
										Mean 68

Table II. *Equilibria in systems containing calcium caseinogenate and calcium chloride.*

	Approximate initial concentrations			Concentrations at equilibrium				Ratios at equilibrium			Percentage dissociation of caseinogenate
	Side I		Side II	Calcium		Chloride					
Exp. No.	Caseinogen %	$Ca(OH)_2$ N	$CaCl_2$ N	I N	II N	I N	II N	$\frac{Ca_I}{Ca_{II}}$	$\frac{Cl_{II}}{Cl_I}$	$\left(\frac{Cl_{II}}{Cl_I}\right)^2$	
5	2·0	0·0100	0·0250	0·0224	0·0132	0·0120	0·0129	1·70	1·07	1·14	29
6	5·0	0·0250	0·0250	0·0362	0·0137	0·0115	0·0137	2·64	1·19	1·41	32
7	5·0	0·0250	0·0125	0·0292	0·0073	0·0056	0·0072	4·00	1·28	1·64	27
8	7·0	0·0350	0·0125	0·0396	0·0077	0·0053	0·0078	5·14	1·47	2·16	33
											Mean 30

Table II shows the results obtained with simple systems containing Ca caseinogenate and $CaCl_2$. The results are similar to those shown in Table I, but the distribution ratios of the calcium and the chloride (the ratio of the latter being squared[1]) show a far greater divergence from one another than do the corresponding sodium and chloride ratios of the previous experiments.

If Donnan's theory holds for the calcium systems, the degree of dissociation of the Ca caseinogenate must be considerably lower than that of the Na caseinogenate: on calculation (by a similar method to that employed above) the percentage dissociation is in fact found to lie between 27 and 33, with a mean value of 30. Such a value agrees well with that obtained from conductivity data by Robertson [1920].

Table III. *Equilibria in simple and in mixed solutions.*

	Approximate initial concentrations					Concentrations at equilibrium						Ratios at equilibrium		
	Side I			Side II		Sodium		Calcium		Chloride				
Exp. No.	Caseinogen %	NaOH N	$Ca(OH)_2$ N	NaCl N	$CaCl_2$ N	I N	II N	I N	II N	I N	II N	$\frac{Na_I}{Na_{II}}$	$\frac{Ca_I}{Ca_{II}}$	$\frac{Cl_{II}}{Cl_I}$
3	5·0	0·025	—	0·025	—	0·0337	0·0145	—	—	0·0087	0·0145	2·32	—	1·67
6	5·0	—	0·025	—	0·025	—	—	0·0362	0·0137	0·0115	0·0137	—	2·64	1·19
9	5·0	0·025	—	—	0·025	0·0142	0·0100	0·0215	0·0035	0·0107	0·0135	1·42	6·15	1·26

In Table III typical results obtained in these simple systems are compared with the results obtained in a mixed system containing Na caseinogenate and $CaCl_2$. It is in this experiment, and in the succeeding experiments, that the very important influence of the degree of dissociation in systems of mixed

[1] To conform to Donnan's equilibrium equation.

electrolytes becomes apparent. It will be seen that the low degree of dissociation of the Ca caseinogenate has caused a preferential absorption of the calcium to the protein side of the membrane, with a consequent high distribution ratio of calcium of 6·15, and lowered distribution ratios of the sodium and chloride of 1·42 and 1·26 respectively.

Table IV. *Equilibria in systems containing sodium caseinogenate, sodium chloride and calcium chloride.*

	Approximate initial concentrations				Concentrations at equilibrium						Ratios at equilibrium		
	Side I		Side II		Sodium		Calcium		Chloride				
Exp. No.	Caseinogen %	NaOH N	NaCl N	$CaCl_2$ N	I N	II N	I N	II N	I N	II N	$\frac{Na_I}{Na_{II}}$	$\frac{Ca_I}{Ca_{II}}$	$\frac{Cl_{II}}{Cl_I}$
1	5·0	0·0250	0·1000	—	0·0688	0·0526	—		0·0438	0·0526	1·31	—	1·20
10	5·0	0·0250	0·0875	0·0125	0·0573	0·0487	0·0100	0·0026	0·0432	0·0513	1·17	1·17	1·17
11	5·0	0·0250	0·0750	0·0250	0·0501	0·0453	0·0197	0·0056	0·0448	0·0509	1·10	3·50	1·13
12	5·0	0·0250	0·0625	0·0375	0·0428	0·0403	0·0279	0·0103	0·0457	0·0506	1·06	2·71	1·11
13	5·0	0·0250	0·0500	0·0500	0·0367	0·0340	0·0347	0·0158	0·0464	0·0498	1·07	2·21	1·07
14	5·0	0·0250	0·0375	0·0625	0·0286	0·0276	0·0420	0·0218	0·0456	0·0494	1·03	1·93	1·07

A more extended study of mixed systems of this type is shown in Table IV. In this series, the concentration of the Na caseinogenate and the total concentration of chlorides have been kept constant, but the relative concentrations of NaCl and $CaCl_2$ have been varied. The following are the salient features of these results.

(*a*) As the total calcium in the system is decreased, an increasing proportion of calcium passes through the membrane. This is to be expected, since (owing to its low degree of dissociation) the smaller the ratio of calcium to caseinogen, the greater will be the relative amount of calcium bound by the caseinogen.

(*b*) As the total calcium in the system is increased, the distribution ratios of the sodium and chloride decrease, until, with high concentrations of calcium, the ratios approach unity. If the reversible reaction

$$\text{Na caseinogenate} + CaCl_2 \rightleftharpoons \text{Ca caseinogenate} + NaCl \quad \ldots(1)$$

is considered, it will be seen that the addition of $CaCl_2$ should tend to force the reaction from left to right, *i.e.* to form additional Ca caseinogenate and NaCl. Hence with increasing calcium in the system, the proportion of undissociated Ca caseinogenate and of freely diffusible NaCl should both increase, and (if Donnan's theory holds good) the distribution ratios of the sodium and the chloride should approach unity. The experimental results confirm this. Actually the distribution ratio of the sodium should always be slightly higher than that of the chloride, since there must always be a small quantity of undissociated Na caseinogenate in the system (the above equation being reversible). The method of analysis was not sufficiently accurate to reveal such a difference. The difference is, however, seen in the results of Exp. 9 of Table III.

Table V. *Effect of varying concentrations of sodium chloride on the distribution of calcium.*

	Approximate initial concentrations				Concentrations of calcium at equilibrium		Ratio at equilibrium
	Side I		Side II				
Exp. No.	Caseinogen %	NaOH N	$CaCl_2$ N	NaCl N	I N	II N	$\frac{Ca_I}{Ca_{II}}$
9	5·0	0·025	0·025	—	0·0215	0·0035	6·15
11	5·0	0·025	0·025	0·075	0·0197	0·0056	3·50
15	5·0	0·025	0·025	0·75	0·0144	0·0101	1·42
16	5·0	0·025	0·025	2·5*	0·0152†	0·0118	1·29

* Contained appreciable quantities of calcium as an impurity; this accounts for the high analytical values obtained.

† Volume of solution reduced to 85 cc., *i.e.* it is actually a 5·9 % caseinogen solution.

It has been shown above that the presence of increasing concentrations of $CaCl_2$ causes a shift in the equilibrium in equation (1) from left to right. An attempt was made to cause the reverse reaction, *i.e.* to shift the equilibrium from right to left, by adding large quantities of NaCl to the system. Table V shows that this attempt was successful. As the NaCl in the system was increased (the quantities of $CaCl_2$ and of Na caseinogenate being kept constant), the proportion of calcium passing through to the protein side of the membrane decreased, until, with very high concentrations of NaCl, the distribution ratio of the calcium itself approached unity.

Discussion.

The above results fully confirm the main conclusions of the previous paper [Wright, 1926]. Further, they demonstrate that the unequal distribution of inorganic salts on either side of the membrane can be fully explained if two factors—the degree of dissociation of the protein salt, and the establishment of a Donnan equilibrium—are taken into account.

It should be understood that the systems studied have not been selected for the purpose of illustrating striking distribution ratios: they have been selected primarily in order to elucidate the factors involved in the establishment of the unequal distribution. Nevertheless the results, together with those previously published, demonstrate the remarkable alterations in the distribution of salts on either side of an artificial membrane caused by the presence of a non-diffusible electrolyte (caseinogen). Further, they demonstrate the marked influence of the presence of one ion on the distribution ratios of other ions; for instance, in the influence of calcium on the sodium and chloride ratios in Exps. 9 to 14, and in the influence of sodium chloride on the calcium ratios in the experiments shown in Table V. It should be emphasised that such alterations in the distribution ratios are entirely independent of the nature of the membrane, provided that the latter fulfils the conditions necessary for the establishment of a Donnan equilibrium, *i.e.* impermeability to one ion of the system but complete permeability to all other ions.

In drawing attention to the more general applications of these results in

problems of cellular physiology, it is necessary to recognise that simple equilibria of the type discussed in this paper can only provide a partial explanation of the remarkable inequalities in the distribution of inorganic elements in living tissues. In the tissues, the various constituents are undergoing continuous transformations, which alter not only the internal phases of the cell but also the nature of the cell membrane itself. Recent papers on permeability have in fact tended to stress the importance of the constitution of the cell membrane in accounting for the accumulation of inorganic elements in living tissues; the membrane has been assumed to have the property of presenting varying resistance to the passage of different inorganic ions.

While such an assumption has in many cases received justification, the results detailed in this paper point to a further explanation, based on the degree of dissociation of the non-diffusible (protein) salts of the cell and on the establishment of a Donnan equilibrium, which may also be of considerable importance in accounting for the unequal distribution of inorganic elements in living tissues. Specific examples of the possible applications of these results may be cited (1) in the experiments of Stiles and Kidd [1919] on the absorption of salts by carrot tissues, (2) in the experiments of Hoagland [1923] on the influence of one salt on the distribution of another salt between the plant and its surrounding medium, and (3) in the experiments of Loeb and Nichols [1926, 1927] on the diffusibility of the calcium of blood-serum. The application of the results to the secretion of calcium in milk has been dealt with elsewhere [Wright, 1928].

SUMMARY.

1. The unequal distribution of salts on either side of an artificial membrane in systems containing caseinogen, NaCl and $CaCl_2$, has been examined.

2. It is shown that the results may be quantitatively explained if two factors, the degree of dissociation of the protein salts and the establishment of a Donnan equilibrium, are taken into account.

3. The influence of one ion on the distribution ratios of other ions is demonstrated.

4. The applications of the results in the general study of the unequal distribution of salts in living tissues are briefly discussed.

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XLIV. THE ABSORPTION OF WATER BY GELATIN.

PART III. THE SULPHATE SYSTEM.

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In two previous papers [Jordan-Lloyd and Pleass, 1927, 1928] the absorption of water by gelatin in the chloride and nitrate systems has been described. The present paper deals with a system containing hydrogen, hydroxyl, sodium and sulphate ions and thus considers the specific effect of the bivalent sulphate ion. The water absorption has again been measured by the percentage change in weight, calculated on the air dry weight at 100°, of pieces of purified leaf gelatin when immersed in the experimental solutions, usually for a period of 3 days. The experimental material and method are the same as previously described with the exception of the important advantage of the use of the glass electrode for the determination of the p_H values. This has markedly decreased the magnitude of the errors occurring in the alkaline zone.

The influence of the three variables, p_H value, temperature (t) and concentration of sodium sulphate (M), on the water absorption of gelatin was investigated and the results are presented graphically.

It will be convenient again to consider separately the behaviour of the gelatin in the four zones of hydrogen ion concentration, centring respectively on p_H 2·6 (acid zone), p_H 10 (alkaline zone), p_H 5 (isoelectric zone), and p_H 7 (neutral zone).

When immersed in distilled water electrically neutral (isoelectric) gelatin absorbs water by imbibition. This water of imbibition may be regarded as penetrating through the system, forming a solid solution in the gelatin gel. It has been shown previously [Jordan-Lloyd and Pleass, 1928, p. 1013] that reduction of the water of imbibition leads to coagulation of the gel. In both acid and alkaline solutions, the gelatin, acting as a weak base or a weak acid, combines with the electrolyte present to form ionisable salts with a non-diffusible ion. This leads to the production of an internal osmotic pressure within the gel which induces further water absorption. This type of swelling is referred to as osmotic swelling. In solutions of salts at about p_H 5 gelatin absorbs more water than the water of imbibition. In these conditions the water absorption occurs by a different mechanism. Both positive and negative

ions of the salt are apparently absorbed equally by the gelatin, causing hydration of the gelatin. This type of swelling is referred to as lyotropic or salt swelling. In solutions of salts at about p_H 7 the absorption of water appears to be influenced both by osmotic swelling and lyotropic swelling.

THE ACID SWELLING ZONE (p_H 1–4).

The water absorption of gelatin at 18°, after immersion in solutions of sodium sulphate from 0 to 1·0 *M* concentration, plotted against the final p_H of the solution is shown in Fig. 1. When no sodium sulphate is present a curve for the swelling of gelatin in sulphuric acid solutions is obtained which is in many respects similar to the corresponding ones for hydrochloric and nitric acids, but which shows marked differences. Hydrochloric and nitric acids induce a maximum swelling of 6500 and 7000 % respectively on the dry weight of the gelatin at p_H 2·6, while the maximum in sulphuric acid is only about 5100 % and occurs at p_H 3·0.

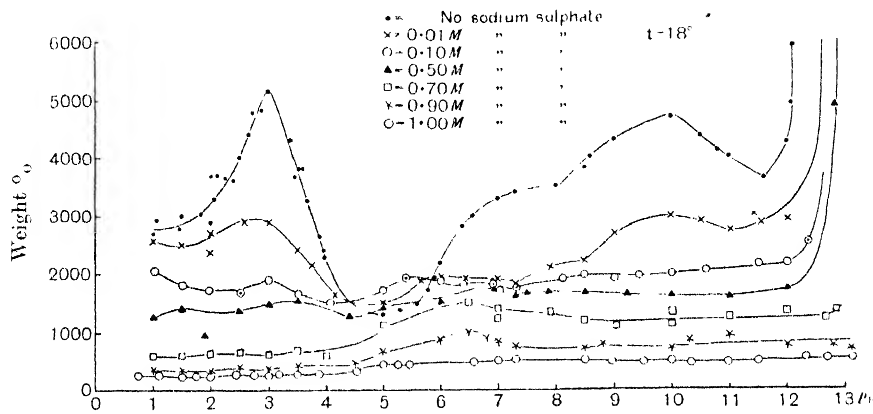

Fig. 1. p_H varying, *t* constant at 18°, *M* constant over a series of concentrations from zero to 1·0 *M*.

It is interesting to observe that from p_H 5·0 to 3·2 the curves for all three acids are similar, that is to say, in dilute solutions at any particular concentration up to 0·001 *N* the water absorption of gelatin will be the same in solutions of all three acids. At greater concentrations, however, the specific nature of the acids becomes apparent. The most arresting observation is that the p_H value of the position of maximum swelling is determined by the anion of the acid. Callow [1925], when measuring the rate of ice crystallisation in a gelatin gel, obtained p_H curves which he compares with Loeb's curves for the swelling of gelatin. The maxima of his ice crystallisation curves occur at p_H 2·5 in hydrochloric acid and at p_H 3·0 in sulphuric acid. Previously it has been stated [Loeb, 1922] that bivalent acids induce swelling which at the maximum is only half as great as that produced by univalent acids. This is not true in the conditions under investigation. The maximum swelling in

sulphuric acid is about 4/5 of the maximum swelling in hydrochloric or nitric acids. At p_H 2·5, however, the increase in swelling above that due to the water of imbibition is about twice as great in hydrochloric and nitric acids as it is in sulphuric, but it must be remembered that this is a comparison at one arbitrary p_H value and not a comparison of the magnitude of the swelling at the various maxima. Kuhn [1922] has measured the increase in volume of powdered commercial gelatin in solutions of hydrochloric, nitric and sulphuric acids. He finds that the maximum in solutions of nitric acid is slightly greater than the maximum in solutions of hydrochloric acid and considerably greater than the maximum in solutions of sulphuric acid. Also

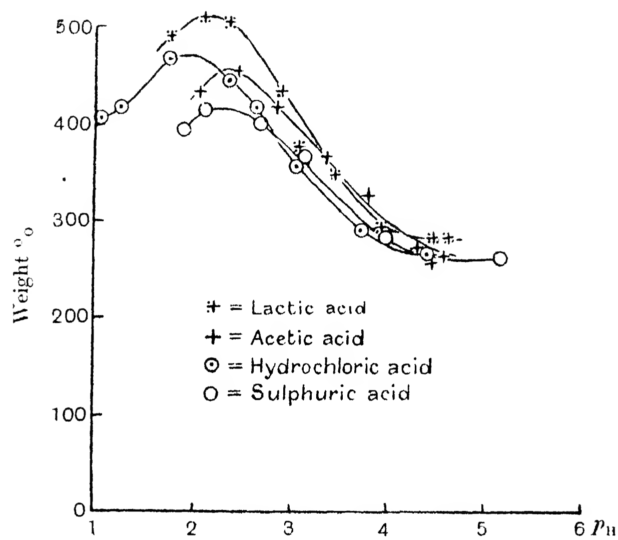

Fig. 2. The water absorption of collagen in lactic, acetic, hydrochloric and sulphuric acids at p_H values between 1 and 5.

Küntzel [1926] has measured the swelling of fibrous collagen by the contraction in length that occurs when the tendons from rats' tails are immersed in acid solutions. He states that nitric acid causes slightly greater maximum swelling than hydrochloric acid, sulphuric acid considerably less. The ratios of the magnitude of maximum swelling in sulphuric, hydrochloric and nitric acids are given in Table I. It is interesting to observe that results obtained by three very different methods indicate that although these monobasic acids induce greater swelling of proteins than sulphuric acid, the ratio of the

Table I. *The ratio of maximum swelling attained in sulphuric, hydrochloric and nitric acids.*

	H_2SO_4	HCl	HNO_3
Increase in weight of pure leaf gelatin (Jordan-Lloyd and Pleass)	1	1·27	1·37
Increase in volume of powdered commercial gelatin (Kuhn)	1	1·17	1·20
Contraction in the tendons of rats' tails (Küntzel)	1	1·26	1·33

maxima is, in every case, much less than 2. The results also confirm the fact that the swelling in nitric acid is only slightly, yet quite definitely, greater than that in hydrochloric acid. This difference in the degree of swelling is probably not due to the valency of the acid but to the nature of the anion.

Jordan-Lloyd and the author (unpublished results) have investigated the swelling of collagen in hydrochloric, sulphuric, lactic and acetic acids (Fig. 2). The experimental material was ox hide which had been limed to remove hair, albumins, globulins and mucoids and then, after neutralisation and washing free from mineral matter, had been dehydrated with acetone. Maximum swelling of collagen in the form of a fibrous tissue occurs in acid solutions at lower p_H values than that of gelatin in similar solutions. The degree of swelling is also much less [Jordan-Lloyd and Kaye, 1924]. The graphs show clearly that not only does the magnitude of the four maxima vary from 420 to 510 % on the dry weight, but that the p_H value at the maximum is specific for each acid. The maximum water absorption of collagen occurs at p_H 2·4 in sulphuric acid and at p_H 1·9 in hydrochloric acid solution. The curves indicate that in solutions of p_H values between 5·0 and 2·8 the water absorption in the presence of both hydrochloric and sulphuric acids is similar. In acetic and lactic acid solutions, however, the curves, even in this p_H range, are slightly removed from those for the inorganic acids, but as here the experimental error is much greater than in the gelatin systems this apparent difference may not be significant. Ostwald, Kuhn and Böhme [1925], when working with powdered gelatin in solutions of organic acids, obtained swelling curves which even for dilute solutions showed marked differences from one another. They state that swelling does not depend on the valency of the acid and that there is no correlation between the volume of maximum swelling in any acid and its ionisation constant.

Isgarischev and Pomeranzeva [1926] have measured the swelling of powdered caseinogen in $N/20$ solutions of 25 organic acids. No record is given of the p_H value of the solutions and the differences observed are certainly partially due to the variation in strengths of the acids. They state that there is no simple numerical relationship between the magnitude of the ionisation constant of the acid and the magnitude of the swelling of the caseinogen and suggest that the predominating factor is the nature and structure of the anion of the acid. In this connection probably one of the most important properties of the acid is its affinity for water. If the acid has a great affinity for water it is very probable that less water will be available for absorption by the gelatin. Fig. 2 shows that lactic acid, which has a tendency to form an anhydride at higher temperatures, *i.e.* an acid which has a negative affinity for water, induces a high degree of swelling, while sulphuric acid, which has a great affinity for water, especially at low p_H values, is, by some mechanism, preventing the gelatin from absorbing the water to so great an extent as in hydrochloric and nitric acid solutions. This view of the factors influencing swelling is supported by the influence of the presence of sodium sulphate,

which also in the anhydrous condition has a great affinity for water. The suppression of the acid swelling due to the presence of 0·01 M sodium sulphate is far greater than the corresponding suppression in the chloride and nitrate systems. When the concentration of the sulphate is increased to 0·5 M all acid swelling is completely suppressed and at greater concentrations of sodium sulphate the gelatin is coagulated to a high degree (see Figs. 1 and 9). This coagulation by sulphuric acid and sodium sulphate is much more intense and far more independent of the reaction of the solution than coagulation in either the chloride or nitrate systems. The pieces of gelatin on removal from M sodium sulphate solutions at all p_H values from 1–12 were very thin and brittle and rapidly dried in air, developing a surface efflorescence of sodium sulphate crystals. This suggests that the nature of the anion in both the pure acid solutions and in the salt solutions plays a very important part in determining the magnitude of the swelling. Also, in the sulphate system (see Fig. 9), when the water absorption at constant p_H values is plotted against the logarithm of the concentration of the sodium sulphate there is a point of inflexion on the curves at the point where the water absorption of the gelatin is equal to that in distilled water. This point corresponds to Loeb's theoretical deduction of the limiting value of the suppression of the swelling when the internal osmotic pressure is zero [Loeb, 1922].

The swelling of gelatin in all concentrations of sulphuric acid becomes greater as the temperature is increased (see Fig. 4). The influence of temperature on the water absorption of gelatin in acid solutions of varying concentrations of sodium sulphate at p_H 3·5 is shown in Fig. 3. It is of importance to note that the temperature coefficient of swelling becomes smaller as the salt concentration is increased. It may also be observed that, as in the chloride and nitrate systems, at all salt concentrations the temperature coefficient of water absorption is much greater above 12° than below this temperature; as this is true of the behaviour of the gelatin in the chloride nitrate and sulphate systems it strongly suggests that it is due to some inherent property of the gelatin and gives support to the theory of the transformation of the gel form (gelatin A) to the sol form (gelatin B), a change which begins at about 15° [Smith, 1919]. In sulphuric acid in the absence of inorganic salts and at reactions more acid than p_H 3·5 the gelatin dissolves at about 22°. Fig. 3 indicates that addition of increasingly large quantities of sodium sulphate not only decreases the swelling but also increases the temperature at which complete solution of the gelatin takes place.

The alkaline swelling zone (p_H 8–13).

At 18° the maximum swelling of gelatin in solutions of sodium hydroxide is 4700 % of its dry weight and occurs at p_H 10·0. As the alkalinity of the solution is decreased there is a rapid decrease in the water absorption until in solutions of p_H values between 8 and 7 the water absorption is nearly

constant and then in solutions of lower p_H value it more rapidly decreases again to reach the minimum at p_H 5.

In the alkaline zone the suppression of the swelling caused by the presence of sodium sulphate is much more pronounced than that caused by the presence of sodium chloride or sodium nitrate (see Fig. 1). Even a concentration of sodium sulphate as low as 0·01 *M* reduces the swelling at the alkaline maximum to 3000 % and 0·1 *M* completely masks the effect of the p_H value of the solution in the entire alkaline zone. In the two previous studies it was observed that sodium chloride reduced alkaline swelling, but at no concentration

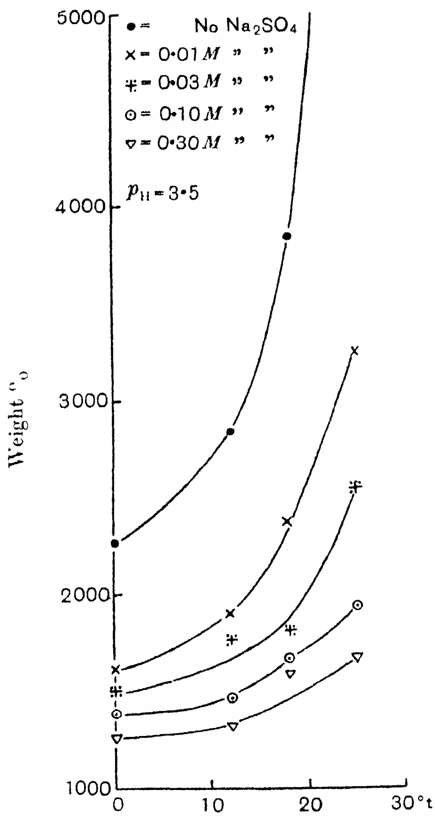

Fig. 3. p_H constant at 3·5, *t* varying from 0° to 25°, *M* constant at 5 values varying from 0 to 0·30 *M*.

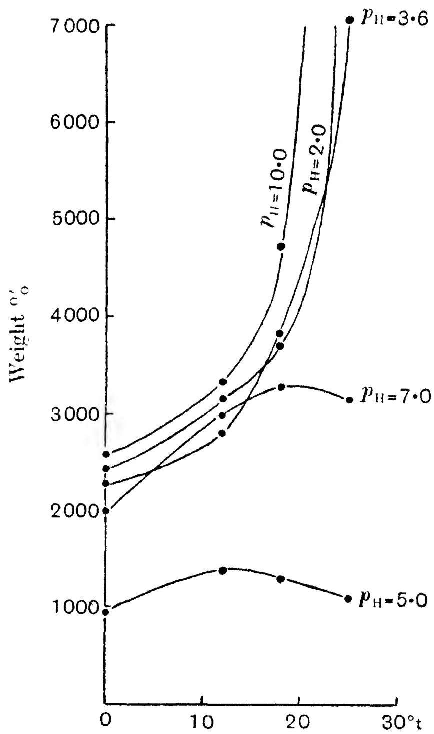

Fig. 4. *t* varying, *M* constant at zero, p_H constant at 2·0, 3·6, 5·0, 7·0 and 10·0.

up to 2 *M* was this salt able to suppress it completely, and that the influence of sodium nitrate was found to present a marked contrast. In solutions of concentrations of 0·1 *M* or less, the swelling was reduced although not entirely suppressed, but above this concentration sodium nitrate, by virtue of its solvent action, caused an increase in the water absorption until at concentrations of nitrate greater than 1·0 *M* the gelatin completely dissolved in solutions at all alkaline reactions. In the sulphate system, however, yet a

third type of salt behaviour is observed. Increasing the concentration of the sodium sulphate leads to a proportionate increase in the suppression of the swelling until at concentrations of sulphate greater than 0·7 *M* coagulation is induced and is intensified as the salt concentration is increased. In molar solutions of sodium sulphate the gelatin is highly coagulated, but not to quite such a high degree as in the corresponding acid solutions. The influence of the addition of sodium sulphate to solutions at p_H 10 on the water absorption of gelatin is shown in Fig. 9. Between values of 0·01 and 0·5 *M* the water absorption is inversely proportional to the logarithm of the salt concentration. At about 0·5 *M* there is a point of inflexion on the curve after which the swelling decreases much more rapidly and coagulation occurs.

The property of sodium sulphate of causing coagulation in moderately concentrated solutions is also accompanied by a reduction in the solvent action of the sodium hydroxide solutions. In pure sodium hydroxide solutions complete solution of the gelatin occurs at p_H 12·1, while in 0·1 *M* sulphate solutions the gelatin dissolves at p_H 12·6 and in 1·0 *M* solutions the gelatin has not dissolved even at p_H 13·1.

THE ISOELECTRIC ZONE (p_H 4–6).

Minimum water absorption of gelatin occurs at p_H 5·0 if the solution is free from salts. The presence of sodium sulphate in fairly dilute solution causes an increase in the water absorption of gelatin in the isoelectric zone. As in the chloride and nitrate systems previously investigated, the salt swelling is proportional to the logarithm of the sodium sulphate concentration at concentrations between 0·01 and 0·1 *M* (see Fig. 9). In more concentrated solutions, however, the three systems exhibit marked differences from one another. In concentrated solutions of sodium chloride the logarithmic relationship continues to be mathematically exact up to 2 *M* concentration. Bechhold [1912] states that there is a maximum swelling induced in isoelectric sodium chloride solutions at 2·4 *M* concentration. In solutions of sodium nitrate more concentrated than 0·1 *M* lyotropic swelling increases more rapidly and is proportional to the nitrate concentration until in molar solutions the gelatin is on the verge of solution. In the sulphate system, however, it will be seen from Figs. 6 and 10 that as the concentration of sodium sulphate is increased from 0·1 to 0·5 *M* the swelling is practically constant, *i.e.* almost independent of the sulphate concentration, but at greater concentrations the swelling is inversely proportional to the salt concentration. Thus in the isoelectric range also, the magnitude of water absorption has been reduced to below that of the water of imbibition and coagulation is again encountered.

The influence of temperature on the water absorption of gelatin in solutions at p_H 5 is shown in Figs. 5 and 6. In the absence of salts, the water absorption increases gradually to a maximum as the temperature is increased from 0° to 12°. Above 12° the water absorption again decreases. In 0·01 *M*

sodium sulphate solution the water absorption increases regularly with temperature but as the sulphate concentration is increased to 0·1 *M* the temperature coefficient becomes increasingly larger at temperatures above 18°. In 0·3 *M* and 0·5 *M* solutions the temperature coefficient of water absorption is quite small. The curve of water absorption in molar sodium sulphate solution shows that less swelling occurs at higher temperatures. It should be noted, however, that the sodium sulphate is much less soluble at low temperatures and that a considerable quantity of the salt has crystallised out at 0°, leaving a solution which is less concentrated than molar.

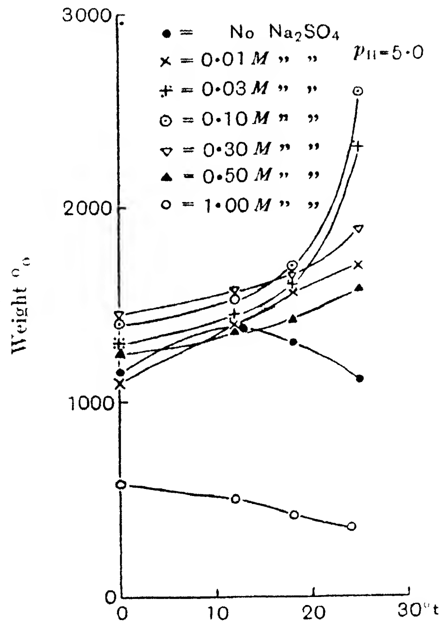

Fig. 5. *t* varying, p_H constant at 5·0, *M* constant at 7 values varying from 0 to 1·0 *M*.

At a temperature of 25° there is a clearly defined maximum of lyotropic swelling in 0·1 *M* sodium sulphate, greater concentrations strongly suppressing the swelling and causing coagulation of the gelatin (Fig. 6). At 0°, 12° and 18°, the maxima are not pronounced, the magnitude of the swelling at each temperature only varying slightly between 0·01 and 0·3 *M* sodium sulphate concentration.

The neutral zone (p_H 6–8).

It can be seen from Figs. 1 and 8 that at 18° the swelling of gelatin in dilute solutions of sodium hydroxide of p_H value between 6 and 8 is reduced by the addition of sodium sulphate in concentrations up to 0·02 *M*. Unlike its behaviour in similar solutions of the chloride or nitrate systems, the gelatin absorbs slightly more water as the sulphate concentration is increased

to 0·3 M after which further increase in the salt concentration leads to rapid decrease in the water absorption and coagulation. The influence of temperature on the water absorption at p_H 7·0 and 7·3 is illustrated by Figs. 7 and 8. In sodium sulphate solutions of concentration greater than 0·02 M, the curves in Figs. 6 and 8 show great similarity at temperatures of 0°, 12° and 18°, indicating that the magnitude of the swelling is similar in corresponding solutions at p_H 5 and 7. At 25°, however, the curve for swelling in solutions at p_H 7 does not exhibit a maximum at 0·1 M sodium sulphate concentration as does the curve for solutions at p_H 5. The water absorption of gelatin in

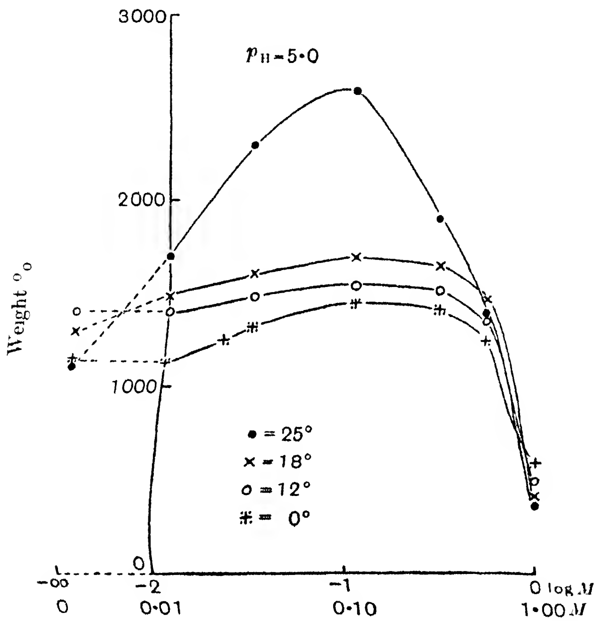

Fig. 6. M varying, p_H constant at 5·0, t constant at 0°, 12°, 18° and 25°.

solutions of varying concentration of sodium sulphate at p_H 7·3 plotted against the temperature is shown in Fig. 7. The maximum of water absorption, which occurs at about 15° in sodium hydroxide solutions of p_H 7·3, is absent when any sodium sulphate is present. The curves relating to all concentrations of sulphate from 0·01 to 0·3 M lie close together and show a gradually increasing temperature coefficient as the temperature is increased. In molar solutions increase in temperature causes decrease in water absorption by the gelatin. This increase in water absorption of gelatin at low temperatures may be affected to some extent by the decreased solubility of the sodium sulphate. In moderately concentrated solutions the sodium sulphate produces the predominating effect.

It can be seen in Fig. 10 that at all p_H values and at all concentrations

of sodium sulphate solutions from 0·1 to 1·0 M the magnitude of the swelling is determined by the sulphate concentration almost independently of the p_H value of the solution.

Discussion.

The behaviour of gelatin in the presence of dilute solutions of sulphuric acid and sodium sulphate is to some extent similar to its behaviour in corresponding solutions in the chloride and nitrate systems, but, even in dilute

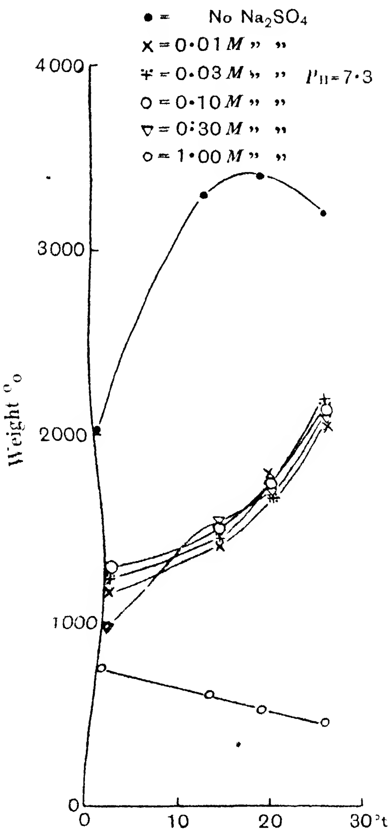

Fig. 7. t varying, p_H constant at 7·3, M constant at 5 values varying from zero to 1·0 M

solutions and to a much greater extent in concentrated ones, marked differences are patent.

From a physico-chemical view-point the three acids and their corresponding sodium salts are similar in many respects but the sulphate ion is differentiated by its bivalent character. Sulphuric acid also is not ionised to so great an extent and is only about half as strong as nitric and hydrochloric acids and has a great affinity for water.

It should be borne in mind when comparing the effect of sodium sulphate

with that of sodium chloride and nitrate that the unit of concentration used is molecular and that equal molecular concentrations do not indicate equal ionic concentrations. This is particularly important when considering osmotic effects. The three systems which have been investigated form a very convenient triad. The hydrochloric acid, sodium chloride, sodium hydroxide system presents what may be regarded as the simplest type, *i.e.* the salt action is almost entirely confined to suppression of osmotic swelling in the acid and alkaline zones and to inducing lyotropic swelling near the isoelectric point.

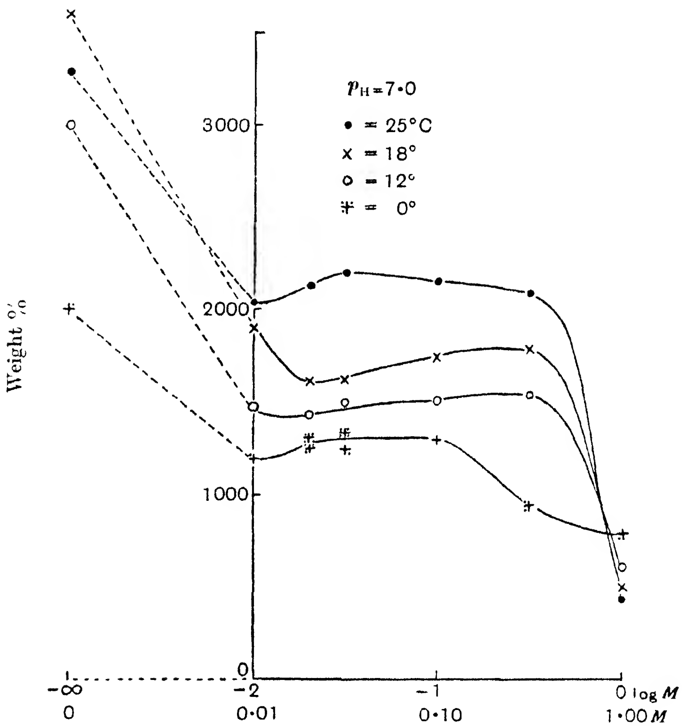

Fig. 8. M varying, p_H constant at 7·0, t constant at 0°, 12°, 18° and 25°.

The behaviour of gelatin in the nitrate system is similar in dilute solutions to that in the chloride system but, as the concentration of the nitrate is increased, proportionately greater swelling is induced which becomes more and more predominant and leads to solution of the gelatin. In solutions of nitric acid the behaviour of gelatin is similar to that in hydrochloric acid, but the magnitude of the water absorption is slightly greater. The suppression of swelling by sodium nitrate in acid solutions and the induction of lyotropic swelling in solutions of 0 to 0·7 M concentration at p_H 5 are slightly greater than the corresponding effects in the chloride system. As the concentration of sodium nitrate is increased, however, this greater lyotropic swelling proves

to be the precursor of solution, which takes place in 2 M solutions at all p_H values greater than 2·5 and in molar solutions at all p_H values above 5. In the nitrate system coagulation of the gelatin may occur to a greater degree than in the chloride system but its occurrence is far more restricted by the

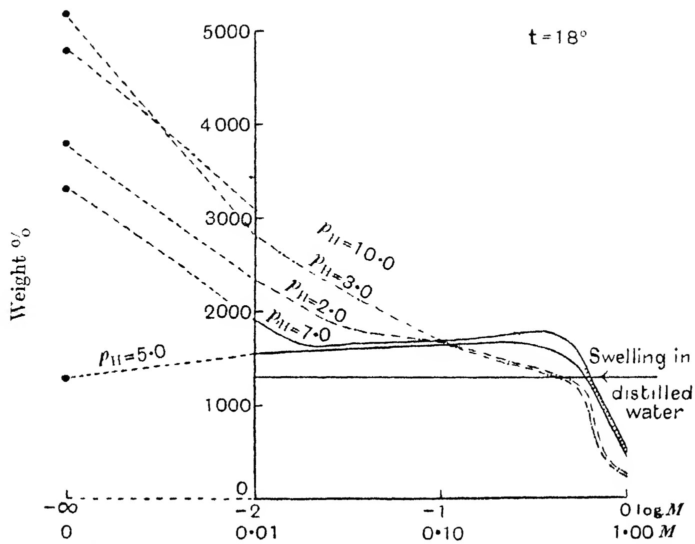

Fig. 9. M varying, t constant at 18°, p_H constant at 2·0, 3·0, 5·0, 7·0 and 10·0

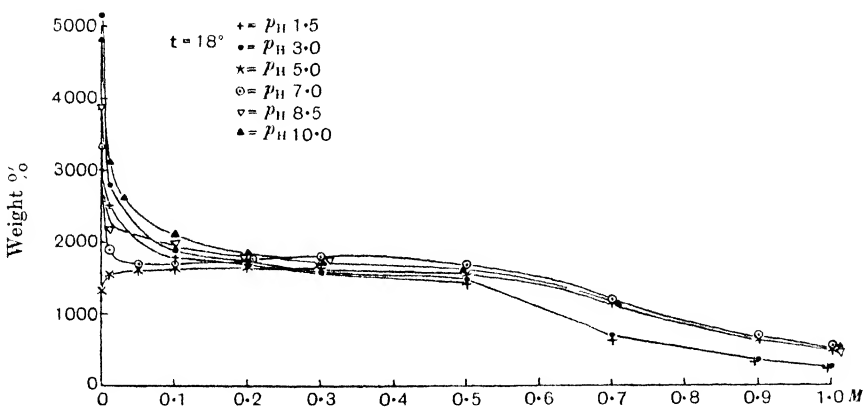

Fig. 10. M varying, t constant at 18°, p_H constant at 1·5, 3·0, 5·0, 7·0, 8·5 and 10·0.

reaction of the solution, due to the fact that the concentrated nitrate solutions exhibit a powerful solvent action. The powerful coagulating action which occurs in sulphate solutions presents a marked contrast. In sulphuric acid solutions of p_H values of 3·0 or less, the magnitude of the swelling of gelatin

is considerably less than in the nitrate and chloride systems. As the concentration of the sodium sulphate solutions at p_H 5 is increased, the lyotropic swelling of gelatin also is suppressed by the coagulating action of the concentrated solutions and in acid solutions the suppression of the osmotic swelling is enhanced. Thus, as the sulphate concentration is increased, coagulation, which is antagonistic to both osmotic and lyotropic swelling, becomes the predominant feature at all reactions.

It has previously been suggested [Jordan-Lloyd and Pleass, 1928] that if coagulation is defined as the reduction of the concentration of water in the gel to below that characteristic of the water of imbibition of the gelatin then the degree of coagulation may be expressed numerically. If the "coagulation ratio" be defined as the ratio of the percentage weight of the gelatin in distilled water to its percentage weight in the conditions under consideration, we may compare the degree of coagulation in different systems. It should be noted that a coagulation ratio of 1 indicates that there is neither swelling nor coagulation. Numbers greater than unity indicate coagulation and less than unity, swelling. In distilled water the swelling of gelatin is 1300 %, while in molar sodium sulphate solution at p_H values from 1–4 it is 250 %. Therefore in these conditions the degree of coagulation is 1300/250 = 5·2. Similarly, at molar concentrations at p_H 2 in the chloride and nitrate systems the coagulation ratio is 1·8 and 2·2 respectively. At p_H 2 in 2 M chloride and nitrate solutions the coagulation is 4·0 and 3·6 units respectively. In molar sodium sulphate solution the coagulation ratio slightly decreases as the reaction becomes more alkaline. At p_H 5 the value is 2·9 units, while at p_H values greater than 7 the coagulation is 2·6 units.

The coagulation in rather more dilute solutions of sodium sulphate is very interesting.

	Coagulation ratio	
p_H	0·9 M	0·7 M sodium sulphate
2·0	2·6	2·0
4·0	2·9	1·9
5·0	2·0	1·2
6·5	1·3	0·8
7·0	1·6	0·9
9·0	1·7	1·1
12·0	1·5	1·0

At p_H 6·5 both the curves for 0·9 and 0·7 M sodium sulphate show a minimum of coagulation.

The curves in Fig. 10 which indicate the influence of salt concentration at various reactions clearly show that although at salt concentrations from 0·1 to 0·5 M the p_H value of the solution has very little effect on the water absorption, at greater concentrations of sodium sulphate, which lead to coagulation of the gelatin, the degree of coagulation is slightly greater in acid than in neutral or alkaline solutions.

It is interesting to observe that coagulation of the gelatin increases its resistance to solution; this is true both of solution caused by increasing the

temperature and solution induced in alkaline fluids of high p_H value. This suggests that coagulation is the converse of lyotropic swelling in this respect also, for swelling appears to precede and promote solution. Thus it appears that the relative concentration of water and gelatin of the aqueous phase of the gel greatly influences the solubility of the gel in water. This suggestion is supported by the fact that dry isoelectric gelatin has a very low solubility in water, even at 100°, whereas if the same sample of gelatin be soaked in water at room temperature for 24 hours, the moist gelatin may then be melted at temperatures above 37° with the formation of quite concentrated solutions (15 % or even greater).

The investigation of the water absorption of gelatin in the sulphate system suggests that in very dilute solutions the action of sulphuric acid and sodium sulphate is very similar to the action of corresponding solutions in the chloride and nitrate systems but as the concentrations are increased the specific nature of the ions becomes more and more the dominating effect. In isoelectric solutions of sodium chloride, nitrate and sulphate as the concentrations of the salts are increased the divergence between the magnitudes of the lyotropic swelling becomes gradually more pronounced. The form of the curves, shown in this and the two previous papers indicating a high degree of swelling leading to solution of the gelatin in the case of the nitrate and coagulation of the gelatin in the case of the sulphate solutions, suggests that with increasing concentration of salt the specific influence of the anions finally overwhelms the influence of the hydrogen and of the hydroxyl ions at all values of p_H.

Summary.

1. Maximum swelling of gelatin occurs in a solution of sulphuric acid at p_H 3·0.

2. Sodium sulphate in concentrations up to 0·5 M in the presence of sulphuric acid suppresses the osmotic swelling of the gelatin due to the acid. At greater concentrations of this salt there is coagulation of the gelatin.

3. Increasing the temperature of solutions in the acid zone causes an increased water absorption by the gelatin. The temperature coefficient of swelling is greater the higher the temperature, but is decreased by an increase in the concentration of sodium sulphate.

4. The osmotic swelling of gelatin in alkaline solutions is suppressed to a greater degree by solutions of sodium sulphate than by equal molar concentrations of sodium chloride or nitrate. At concentrations of sodium sulphate of 0·7 M or greater, coagulation is induced.

5. At the isoelectric point of gelatin the water absorption in solutions of sodium sulphate is proportional to the logarithm of the salt concentration up to 0·1 M. At greater concentrations lyotropic swelling is reduced and coagulation occurs in solutions of greater than 0·7 M concentration.

6. In solutions of sodium sulphate of 0·1 to 1·0 M concentration the magnitude of the water absorption of the gelatin is determined chiefly by the sulphate concentration almost independently of the p_H value of the solution.

7. A method of interpreting the term "coagulation" on a numerical scale is described.

8. Gelatin in the coagulated condition tends to resist the solvent action of alkaline solutions.

9. Increase in temperature generally causes greater water absorption, the temperature coefficient becoming larger as the temperature is increased.

In conclusion, the author wishes to thank the Council of the British Leather Manufacturers' Research Association for permission to publish this paper and to express her gratitude to the Director, Dr Jordan-Lloyd, for her advice and helpful criticism during the progress of this work.

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XLV. OBSERVATIONS ON THE IODINE-CONTAINING COMPOUNDS OF THE THYROID GLAND. ISOLATION OF *dl*-3:5-DI-IODOTYROSINE.

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About three years ago one of the authors [Harington, 1926] described an improved method for the isolation of thyroxine from the thyroid gland, which was based on a graduated hydrolysis of the gland substance with barium hydroxide. When desiccated thyroid was boiled with dilute barium hydroxide the iodine was separated into three fractions, the first being contained in the precipitate of insoluble barium salts, the second being obtainable by acidification of the alkaline filtrate, and the third remaining soluble in presence of acid. Thyroxine was isolated by subjecting the first two fractions to more intensive hydrolysis with barium hydroxide, whilst no evidence could be obtained of the presence of thyroxine in the third, acid-soluble, portion. A similar separation of the iodine compounds of the thyroid into acid-soluble and acid-insoluble had been effected previously by Kendall by hydrolysis with dilute sodium hydroxide, and he was able to show [1919] that the physiological activity was confined to the acid-insoluble fraction, *i.e.* to that fraction from which alone thyroxine could be isolated. This observation has been confirmed by an experiment kindly performed for us by Dr J. H. Gaddum. Desiccated thyroid gland was boiled with 10 % crystalline barium hydroxide under the usual conditions and the hydrolytic products were separated into acid-insoluble and acid-soluble fractions; injection of 2·6 mg. per kg. of the iodine of the acid-insoluble portion produced an increase of 60 % in the oxygen consumption of a rat, whilst a definitely smaller effect was obtained after injection of 26 mg. per kg. of the iodine of the acid-soluble fraction. It is evident, therefore, that this preliminary hydrolysis, although it is of so mild a character that it cannot conceivably involve any destruction of thyroxine, effects a practically complete separation of the latter from the other iodine-containing compounds which may be present.

At the time of the previous investigation the isolation of thyroxine itself was the exclusive object in view and the further examination of the acid-soluble fraction was therefore postponed. The present communication deals

with the results of this examination, leading eventually to the isolation of 3 : 5-diiodotyrosine, and we shall adduce evidence in support of the view that the whole of the acid-soluble iodine is indeed present as diiodotyrosine, whilst the whole of the acid-insoluble iodine belongs in all probability to thyroxine.

If the alkaline filtrate obtained after hydrolysis of desiccated thyroid gland with 10 % barium hydroxide be carefully acidified with hydrochloric acid (to about p_H 5·0) the thyroxine-iodine is, as previously indicated, almost quantitatively precipitated; after removal of this precipitate one is left with a solution containing 50 % or more of the original iodine of the gland, practically the whole of which remains in organic combination. The solution so obtained is, however, of little use for further investigation, owing to its content of barium chloride. Our first step was, therefore, to substitute sulphuric acid for the hydrochloric acid formerly used for the precipitation of the thyroxine fraction. This step resulted in the removal of a certain proportion of the acid-soluble iodine by adsorption on the precipitate of barium sulphate and thyroxine; this amount could be easily recovered, however, by extraction of the precipitate with alkali, removal of the barium sulphate, and acidification, when the thyroxine was again precipitated, and the filtrate containing the acid-soluble iodine which had been removed by adsorption on the barium sulphate could be reunited at a later stage with the main acid-soluble fraction.

To return to the latter, in the first experiment, the solution, which gave a strong biuret reaction and evidently contained for the most part peptones and other higher protein degradation products, was concentrated and subjected to more intensive hydrolysis either with acid or alkali; hydrolysis with acid was soon dismissed as useless, since it resulted in the entire destruction of the organic iodine compounds, the iodine appearing in the solution of hydrolytic products as iodide. Hydrolysis with barium hydroxide was evidently much more favourable since a large proportion of the iodine remained in organic combination; no success, however, attended attempts to isolate the iodine compound from the hydrolytic products. Nevertheless these early experiments were useful in that they gave an indication of the probable nature of the compound for which we were searching; for it was observed that, after partial separation of the hydrolytic products, those fractions which were rich in iodine gave the colour reaction with nitrous acid and ammonia which is given also by thyroxine, and which was previously shown [Harington and Barger, 1927] to be characteristic of the *o*-diiodophenolic grouping, and was put on a roughly quantitative basis by Ingvaldsen and Cameron [1926]. At this stage we returned to the original solution to see whether a preliminary partial separation of the iodine compound might not be effected at this early stage, and the mixture of products obtained after the second hydrolysis be thereby simplified. We found that precipitation with basic lead acetate served our purpose in this respect. Addition of basic lead acetate to the neutralised solution until no further immediate precipitate was formed removed about 80 % of the iodine; the precipitation of the iodine could indeed be made

quantitative by making the solution alkaline with ammonia, but the last 20 % of the iodine was accompanied by so large a preponderance of other material that we found ourselves finally in no better position than before the lead treatment; we therefore contented ourselves with the 80 % precipitation obtained as indicated above. The lead salts were filtered off and decomposed with sulphuric acid, and the filtrate, after removal of sulphuric acid, was united with the portion recovered, as described above, from the first acid precipitate, and was concentrated and further hydrolysed with 40 % barium hydroxide. After removal of barium with carbon dioxide the solution was treated with silver nitrate which precipitated the whole of the iodine; the silver salts were extracted with dilute nitric acid, the iodide formed during the hydrolysis being left undissolved, and were reprecipitated with ammonia; they were then decomposed with hydrogen sulphide in the usual way. We thus obtained a solution which gave a strong nitrous acid reaction, and it became more and more evident that the properties of the substance under investigation were closely similar to those of diiodotyrosine.

Earlier workers who have isolated this compound from natural sources [cf. *e.g.* Wheeler and Mendel, 1910] have employed hydrolysis with barium hydroxide followed by silver precipitation, and have then utilised phosphotungstic acid in order to separate the diiodotyrosine from the dicarboxylic amino-acids which are the chief accompanying impurity. In our hands this reagent did not prove satisfactory either from the point of view of completeness of precipitation of the iodine, or from that of cleanness of separation. We therefore had recourse to extraction of the neutralised solution with butyl alcohol for the purpose in view. Continuous extraction with this solvent is undesirable, since, on prolonged boiling with butyl alcohol, diiodotyrosine appears to undergo some decomposition; by shaking out the warm solution with successive quantities of butyl alcohol [cf. Onslow, 1921] we were, however, successful in extracting the greater part of the iodine with no disadvantageous effects. The aqueous solution of the material extracted by butyl alcohol still contained some colloidal substances which inhibited the crystallisation of the diiodotyrosine; these substances could be removed by treatment, under the appropriate conditions, with uranium acetate, and then, after one more precipitation as the lead salt, the diiodotyrosine could be obtained crystalline without difficulty.

Reference to the experimental part below, and to the diagrammatic representation of a typical experiment, will show that, although the actual quantity of diiodotyrosine isolated amounts only to about 11 % of the total iodine of the thyroid, the losses during the process of isolation are essentially the continuous small losses of material which are inevitably associated with a somewhat complex manipulation of this type. An exception to this statement may at first sight appear to be indicated by the large loss of iodine as iodide during the intensive hydrolysis with barium hydroxide; we have satisfied ourselves, however, that even pure diiodotyrosine admixed with a protein

and subjected to similar treatment, loses a considerable proportion of its iodine as iodide, and such loss may well be greater when the compound is at the same time being split off from peptide combination; we do not, therefore, regard this phenomenon as invalidating our general argument that at no stage is there any sharp break in the recovery of the iodine, such as would indicate the presence of an iodine-containing compound of a different character. In order further to confirm this conclusion we carried out, in one experiment, colorimetric determinations by the nitrous acid reaction coincidently with our organic iodine determinations, and, although no pretence is made that the colorimetric results were more than approximations, a definite parallelism between the two sets of observations was apparent[1].

Having then convinced ourselves that we could account for all the acid-soluble iodine as diiodotyrosine, we returned to the acid-insoluble fraction, to see whether we could find here any indication of the presence of an organic iodine-containing compound other than thyroxine. In the method previously described [Harington, 1926] the thyroxine was recovered by extraction with alkaline sodium sulphate; this manipulation is troublesome, and it has been found better to decompose the salts by suspension in warm dilute hydrochloric acid; lipoidal material is then removed with ether, and the remaining insoluble precipitate is united with the main acid precipitate for the second hydrolysis. It was formerly stated that, at the end of the second hydrolysis, the whole of the thyroxine was to be found in the precipitate of barium salts; this remark requires modification. The distribution of thyroxine at this stage depends on the concentration of iodine in the solution: if this concentration (at the commencement of the hydrolysis) is, as in the earlier experiments, 1 mg. per cc. or more, it is true that most of the thyroxine appears in the precipitate, although even here traces may be obtained by acidification of the mother liquor; on the other hand, with more dilute solutions, a large part of the thyroxine may remain in solution; in any case, in carrying out this isolation, the solution should at this stage be acidified and any precipitate which is obtained should be combined with the material obtained on recovery from the insoluble barium salts; the combined acid-insoluble material is then converted into crystalline thyroxine by the method previously described [Harington, 1926].

Reference to the experimental part will show that here again we meet, in the process of isolation of thyroxine, with a series of losses such as can

[1] The quantitative nitrous acid reaction in coloured solutions is conveniently carried out as follows. Into each of two test tubes A and B is introduced a 5 cc. sample of the solution to be tested containing approximately 0·5 mg. of iodine per cc. To A is added 1 cc. of a 0·1 % solution of diiodotyrosine, to B 1 cc. of water. To both tubes are added two drops of 30 % sodium nitrite and 3 drops of concentrated hydrochloric acid; the tubes are shaken and allowed to stand 2 minutes at the ordinary temperature; to each is added 9 cc. of butyl alcohol, the contents being shaken for 1 minute and allowed to separate; 5 cc. of each butyl alcohol layer are transferred to the cups of a colorimeter and 0·5 cc. alcoholic ammonia (0·5 N) added; the resulting pink colours are compared immediately.

reasonably be ascribed to unavoidable imperfections of technique. In the particular experiment described, the thyroxine ultimately isolated represented 16 % of the total iodine of the gland substance; this is an average result with the improved technique now employed; we have had even better yields. As in the case of diiodotyrosine, so with thyroxine, the greatest loss occurs at the stage of the intensive alkaline hydrolysis. This loss consists in part of iodine split off as iodide, and for the rest of iodine which is still in organic combination but is soluble in acid. The proportion which appears in the inorganic condition is considerably less than is the case at the corresponding stage in the isolation of diiodotyrosine; this difference, we think, is due to the fact that a great part of the thyroxine, as it is set free by hydrolysis, separates as the sparingly soluble barium salt and is thus removed from the action of the alkali, whilst the soluble diiodotyrosine remains exposed to this action throughout the experiment. As to the further small amount of iodine which appeared at this stage in the acid-soluble organic form (an amount so small as to preclude its detailed examination) two obvious possibilities suggest themselves; in the first place it is naturally not claimed that the division of the products of the preliminary hydrolysis of the thyroid into those containing thyroxine on the one hand and those containing diiodotyrosine on the other is absolutely quantitative; indeed the experiment of Dr Gaddum mentioned above shows that this is not the case, since a trace of residual physiological activity was found in the acid-soluble fraction; if, then, traces of thyroxine may appear among the acid-soluble products, it is equally possible that traces of diiodotyrosine may appear among the acid-insoluble substance; especially does this seem likely to us since one of our principal difficulties in the isolation of diiodotyrosine has been the extreme ease with which, from neutral or slightly acid solutions, this substance is adsorbed on precipitates; it is not unreasonable, therefore, to suppose that part of the fraction of the iodine under consideration belongs in fact to diiodotyrosine. A second possibility which must not be ignored is that, under the somewhat severe conditions of the hydrolysis, a part of the thyroxine may yield a product still containing organically-combined iodine but soluble in acid. Thus it is only those two iodine atoms which are situated *ortho* to the phenolic group of thyroxine which we should expect to be labile towards alkali of the concentration employed during the hydrolysis; were hydrolysis of these two iodine atoms to occur we should have a pyrogallol derivative still containing iodine, which would almost certainly be fairly soluble in water, and such part of which as was not further oxidised would go to make up the fraction which we are discussing. In any case we find it more reasonable to suppose that this small fraction of acid-soluble organic iodine (which is, after all, only 5 % of the total iodine) appears at this stage for some such reason as we have suggested, rather than that it indicates the presence of an independent iodine-containing compound in the original gland.

On the basis of this investigation, therefore, we feel justified in advancing

the definite view that there are only two iodine-containing compounds in the thyroid gland, namely thyroxine and 3 : 5-diiodotyrosine; in the gland which we have used for our experiments, the iodine would appear to be about equally distributed between these two compounds. This gland material, which we have employed throughout, is all obtained from one geographical source, and it has, moreover, shown a remarkable constancy of iodine content at whatever season of the year it has been purchased. There is, however, little doubt that variations in the distribution of iodine in the thyroid between diiodotyrosine and thyroxine may occur. Such variations would account for the lack of parallelism between iodine content and physiological activity which has been observed in the past for different samples of thyroid, and for the observations of Kendall and Simonsen [1928] and others on the varying proportion of the iodine which can be obtained in the acid-insoluble condition; it has indeed been stated that in some glands the iodine is entirely in the acid-soluble condition. The suggestion has already been advanced [Harington and Barger, 1927] that, biologically, thyroxine is derived from tyrosine through the stage of 3 : 5-diiodotyrosine, two molecules of which may be supposed to undergo oxidative coupling with the loss of one side-chain to give thyroxine; the actual isolation of diiodotyrosine from the thyroid lends strong support to this theory. If we are to regard diiodotyrosine as the precursor of thyroxine, it is evident, that with varying states of activity of the gland we must expect to find varying relationships between the amount of the precursor and of the complete hormone. Taking the possibility of such variations into account, an obvious corollary is that the only reasonable chemical assay of the therapeutic value of a thyroid preparation must be based on the acid-insoluble iodine and not on the total iodine content of the material.

Brief reference must here be made to a publication by Kendall and Simonsen [1928] which has recently appeared. These authors cite their own observations and those of other workers to the effect that the whole of the physiological activity of the thyroid gland cannot be accounted for by its thyroxine. The discrepancy to which Kendall draws attention is, however, not so much between the activity of a gland and its content of thyroxine, as between the activity and the amount of thyroxine which can be isolated. We think that most workers who have had experience of this type of manipulation will agree that a very grave difference exists between the amount of a compound which is present in a tissue and the amount of that compound which can be isolated in the pure condition. We have attempted above to bring forward evidence that the whole of the acid-insoluble iodine of the thyroid may be regarded as thyroxine; if this conclusion can be accepted and if we bear in mind that the acid-insoluble fraction usually represents 40 to 60 % of the total iodine, and further, that the true physiological activity of thyroxine as it occurs in the thyroid may well be different from that of the free compound, the discrepancy no longer appears so serious. It may be recalled that the physiological activity of laevorotatory thyroxine was found to be

definitely greater than that of the dextrorotatory isomeride, whence it was assumed that the former was probably the naturally occurring compound [Harington, 1928]. Recent experiments in this laboratory have cast some doubt on the correctness of this deduction, and, until the point is finally cleared up, we do not wish to base any arguments on the optical activity of the naturally occurring hormone. There remains, however, the possibility that the physiological activity of thyroxine in the natural state, *i.e.* in peptide or other form of combination, may be enhanced with respect to its activity in the free condition. We suggest that, in the present state of our knowledge, any remaining discrepancy between the physiological activity and the acid-insoluble iodine content of the thyroid may be explained on these lines more acceptably than by the gratuitous assumption of the existence in the gland of an "active" form of thyroxine differing in chemical structure from the compound as we know it.

Experimental.

In this part a detailed description is given of one complete experiment which is typical of several; for the sake of clarity a diagrammatic representation of the process is given.

Desiccated thyroid gland (250 g. containing 1·220 g. of iodine) was boiled under a reflux condenser for 6 hours with 2500 cc. of a 10 % solution of crystalline barium hydroxide; after cooling, the solution was filtered, and the filtrate brought to p_H 5·0 by addition of 50 % sulphuric acid; the precipitate of thyroxine and barium sulphate, which carried down also some of the acid-soluble iodine by adsorption, was filtered off, and the filtrate and washings (A) containing 515 mg. of iodine were set aside.

(1) *Isolation of thyroxine.* The precipitate of insoluble barium salts resulting from the preliminary hydrolysis was ground up and suspended in dilute hydrochloric acid; the solution was brought to the boil, adjusted to p_H 5·0 and filtered; the precipitate was ground up with ether, again filtered, and dissolved in dilute sodium hydroxide; the resulting solution was combined with that resulting from extraction of the barium sulphate-thyroxine precipitate with warm dilute sodium hydroxide followed by removal of the barium sulphate; the combined sodium hydroxide solutions contained 549 mg. of iodine. The total loss up to this stage was therefore 156 mg. of iodine; this was accounted for in part by a small unhydrolysed residue of the original material, and, for the rest, was spread over the various operations above described; in numerous experiments the distribution of these losses, as between the different operations, was uniform, and each individual loss was small. The sodium hydroxide solutions were acidified with sulphuric acid to p_H 5·0 and the precipitate was filtered off; the solution was treated with slight excess of barium hydroxide and the filtrate (B) containing 118 mg. of iodine was reserved (see below). The acid-insoluble precipitate (431 mg. of iodine) was dissolved in 300 cc. of water with the aid of a little ammonia,

Table 1. *Separation of iodine into acid-soluble and acid-insoluble fractions.*

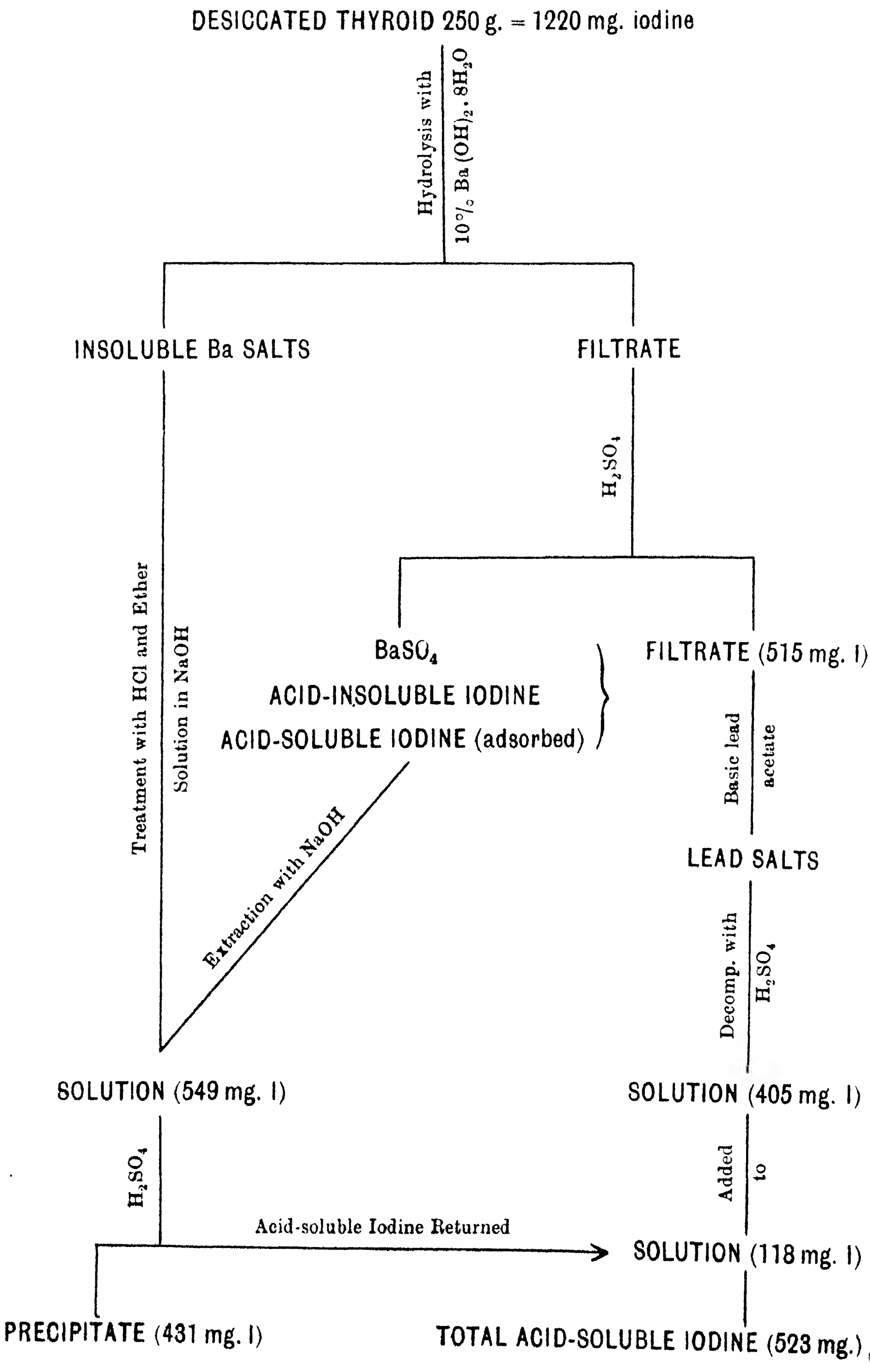
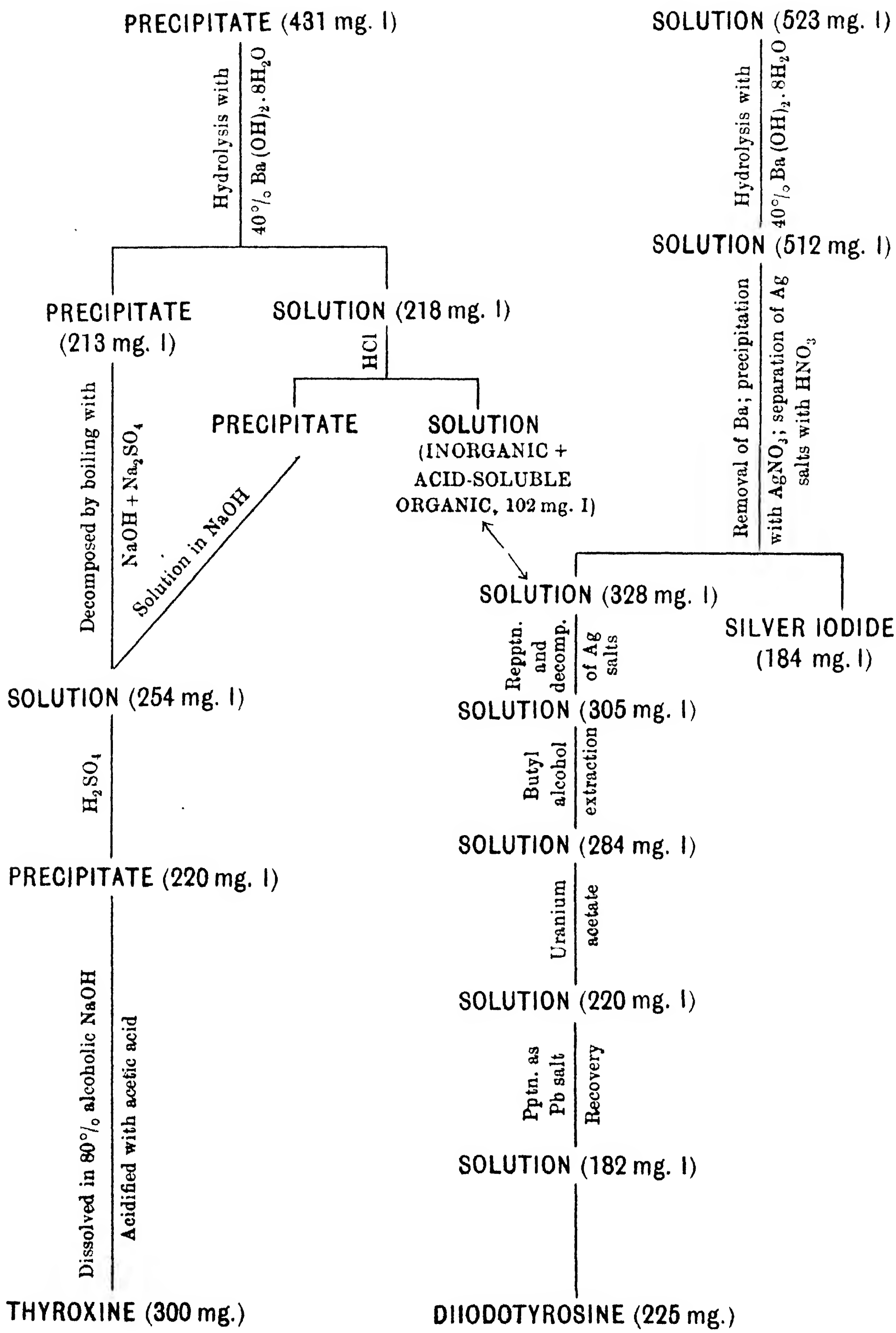


Table II. *Isolation of thyroxine and of diiodotyrosine.*



crystalline barium hydroxide was added to 40 % concentration, and the whole was heated for 18 hours at 100°; the solution was filtered hot, the filtrate cooled and the barium hydroxide which separated was removed; hydrochloric acid was then added to p_H 5·0 and the precipitate (C) collected; the hydrochloric acid filtrate contained 102 mg. of iodine (in a parallel experiment this was shown to consist to an almost equal extent of inorganic iodide and acid-soluble organic iodine). The insoluble barium salts were decomposed by boiling with alkaline sodium sulphate, and to the alkaline filtrate was added the hydrochloric acid precipitate (C); the alkaline solution was heated to boiling and brought to p_H 5·0 with sulphuric acid; the precipitate was collected and dissolved in 80 % alcohol with the aid of sodium hydroxide; the solution was filtered, heated to boiling, and acidified with acetic acid, when there separated 300 mg. of thyroxine. The serious loss in this process occurs therefore during the drastic hydrolysis; the remaining loss is accounted for by the additive effect of incomplete recovery from the insoluble barium salts, incomplete re-precipitation, and finally incomplete separation on crystallisation from acetic acid-alcohol, each individual loss again being small in amount.

(2) *Isolation of dl-3 : 5-diiodotyrosine.* The filtrate A (see above) was treated with basic lead acetate (the B.P. solution) until no further immediate precipitation occurred; after standing overnight the lead salts were filtered off and suspended in water (2000 cc.); the mixture was heated to boiling and 50 % sulphuric acid was added until the reaction remained acid to Congo red; lead sulphate was removed by filtration and the filtrate was freed from sulphuric acid by addition of a slight excess of barium hydroxide. The alkaline filtrate from the barium sulphate contained 405 mg. of iodine, 20 % of the iodine thus having been sacrificed at the stage of lead precipitation for reasons already given. The alkaline filtrate was combined with solution B (see above), and the whole, containing in all 523 mg. of iodine, was boiled down to 500 cc.; 200 g. of crystalline barium hydroxide were added, and the solution was heated for 18 hours at 100°. A small greenish precipitate was filtered off hot, but contained no significant amount of iodine; the filtrate (512 mg. of iodine) was cooled, the barium hydroxide was filtered off and recrystallised; the combined mother liquors were treated with carbon dioxide and the barium carbonate removed by filtration and well washed; the filtrate and washings contained 512 mg. of iodine. Silver nitrate (20 % solution) was now added until precipitation was complete, the mother liquor being free from iodine; the silver salts were filtered off and ground up with dilute nitric acid (free from nitrous acid); the filtered solution contained 328 mg. of iodine, indicating a loss at this stage of 184 mg. as iodide. The organic silver salts were re-precipitated by the careful addition of ammonia, filtered off, washed, suspended in water and decomposed with hydrogen sulphide; the solution was brought to the boil, the silver sulphide removed by filtration, and the filtrate, containing 305 mg. of iodine, was concentrated to 200 cc. under diminished pressure. The solution was now extracted by shaking out nine times with

butyl alchohol (previously purified by agitation with saturated sodium bisulphite followed by distillation) at a temperature of about 70°; the combined butyl alcohol extracts were evaporated to dryness under diminished pressure and the residue dissolved in water; the solution contained 284 mg. of iodine; the volume was made up to 500 cc., the solution was brought to the boil and treated with uranium acetate solution in slight excess; the precipitate was filtered off and the filtrate freed from uranium with ammonia and concentrated to 320 cc. under diminished pressure; it now contained 220 mg. of iodine; basic lead acetate solution was added to complete precipitation, and after standing overnight the precipitate was collected, well washed, and decomposed by saturating its suspension in hot water with hydrogen sulphide; the lead sulphide was boiled out with much hot water, and the filtrate and washings were concentrated to a small volume under diminished pressure; the solution, which was faintly acid to Congo red, was exactly neutralised to litmus with ammonia; on further concentration in a vacuum desiccator over sulphuric acid 225 mg. of a crystalline compound separated out. On recrystallisation from 50 % acetic acid it formed pale straw-coloured prismatic needles having M.P. 198·4° (decomp.); a sample of *dl*-3 : 5-diiodotyrosine, prepared by iodination of *dl*-tyrosine, had M.P. 197·5°, whilst a mixture of the natural and synthetic products melted at 198·0°[1]. The product from the thyroid gave the colour reaction with nitrous acid and ammonia with intensity; on evaporation of a small amount on the water-bath with concentrated hydriodic acid, a residue was left which gave a strong Millon's reaction.

Analysis. 2·168 mg. required 5·00 cc. $N/200$ thiosulphate [Kendall, 1914]
20·3 mg. gave 0·577 mg. N (Micro-Kjeldahl)
20·3 mg. gave 1·035 cc. moist N_2 at 21·5° and 764 mm.

	I	N
Found:	58·2	2·8, 2·9
Calc. for $C_9H_9O_3NI_2$:	58·6	3·2

There remained, therefore, no doubt as to the identity of the compound isolated from the thyroid gland with *dl*-3 : 5-diiodotyrosine.

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XLVI. OBSERVATIONS ON INSULIN.

PART I. CHEMICAL OBSERVATIONS.

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PART I.

Since the first announcement by Abel [1926] of the isolation from commercial insulin of a crystalline substance exhibiting in a high degree the specific activity of the hormone, it has become a matter of the greatest interest to attempt to determine whether this crystalline material is indeed to be regarded as insulin itself, or whether it is in reality an inert constituent of the pancreas which, in the process of preparation, has become associated with the hormone by adsorption or otherwise. The final proof of the identity of the compound with insulin, if such identity exists, can naturally only be afforded by synthesis; since, however, we have to do with a substance which is to be classed among the simpler proteins the prospects of such a synthesis remain infinitely remote, and other, less direct, evidence bearing upon the question must be sought. Later papers from Abel's laboratory [Abel *et al.*, 1927; Jensen and Geiling, 1928; du Vigneaud *et al.*, 1928] have not increased the volume of such evidence to any considerable extent. Slight modifications have been introduced into the original method of preparation of the crystalline material, but these modifications do not successfully meet the criticisms which may be levelled at the claim to designate the substance pure insulin. In particular have the workers in Abel's laboratory failed to meet the criticism based on the varying degree of physiological activity reported for different samples of a supposedly uniform substance. If we turn to the experience of other workers with this material we find that a similar state of affairs seems to exist, for whilst there is no doubt that crystals identical with those

described by Abel have been prepared in other laboratories by his method, no consistency is to be found in the degree of physiological activity assigned to them by different workers, or indeed sometimes by the same worker. It has even been stated [Dingemanse, 1928] that it is possible by adsorption on charcoal followed by elution with phenol and extraction of the material recovered from phenol with phosphate to obtain an amorphous preparation of a higher degree of activity than that ascribed by Abel to the crystals; this statement is, however, contested by the workers in Abel's laboratory [du Vigneaud *et al.*, 1928].

The present uncertain position of the problem seemed to us to warrant a renewed attempt to obtain more decisive evidence upon the point at issue, and we therefore set out on the present investigation with three main objects in view: (*a*) to obtain the material by Abel's method and to subject it to thorough physiological assay, (*b*) to seek for alternative methods of preparation, and (*c*) to carry out certain experiments with the crystalline substance designed to test the question of its identity with insulin.

As starting material in this work we have employed in the main two commercial preparations of insulin. The earlier experiments were made with a powder prepared at the Connaught Laboratories in Toronto and assayed at 13 international units per mg., and the later experiments with a powder of approximately similar unitage kindly supplied to us by the British Drug Houses, to whom, and in particular to Dr F. H. Carr, we wish here to express our best thanks. We have also had the opportunity to make a few experiments with a powder prepared by Messrs Burroughs Wellcome and assayed at 21 international units per mg., for which we are indebted to Dr H. A. D. Jowett. Our first experiments consisted in attempts to prepare crystalline material from the Toronto insulin by the method described by Abel. After several failures, we were eventually able to obtain a small amount of the crystals by this method; our experience with the technique was discouraging, however, since we found it necessary to carry out many more reprecipitations than are indicated by Abel, with the result that the manipulation became excessively laborious and the yields of crystalline material were exceedingly small. As soon, therefore, as we had accumulated sufficient material for the purpose of physiological assay, we passed on to the second object of our investigation, namely the search for an alternative method of preparation.

The first impression which we, and apparently Abel himself, formed (although subsequent work has caused us to modify this view) was that the success of Abel's method depended on the efficient buffering achieved by the complex mixture of weak bases which he employed; it was thought that such a system enabled the reaction of the solution to be adjusted so gently and so exactly to the isoelectric point that it was possible in effect to obtain a supersaturated solution from which the protein might be expected to separate slowly, with consequently increased tendency to crystallise. With this idea in mind we sought to substitute a simple phosphate buffer for the mixture

of brucine acetate and pyridine employed by Abel, the insulin being dissolved at a slightly alkaline reaction, and the p_H of the solution adjusted by the very gradual addition of carbon dioxide; even the very slowest introduction of the latter, however, caused an immediate amorphous precipitate to form and by no fractionation on these lines were we able to obtain crystalline material. In the light of our subsequent experiments we are inclined to think that the function of the brucine acetate-pyridine mixture is not merely, as we first supposed, that of a delicate buffering system, but that other physical properties of these substances are involved, *e.g.* the lowering of the surface tension of the solution, thus bringing into play an effect on the properties of the commercial insulin analogous to that about to be described. Some such explanation of the rôle of brucine is indeed suggested by Abel himself.

The method for the preparation of the crystalline material which has met, in our hands, with a certain amount of success, and which we wish to describe in this paper, rests on the observation that the properties of commercial insulin are profoundly modified by the presence in the solution of an active saponin. As to what exactly constitutes an "active" saponin from this point of view we shall have more to say later; at this point let us assume that we are speaking of one which has been found by experiment to be satisfactory for the purpose in hand. It is well known that the commercial purification of insulin consists, in its final stages, of repeated precipitations at the iso-electric point, the latter lying at about p_H 4·8–5·0; a good commercial product, such as either of those which we have used in this work, precipitates practically quantitatively when the solution is adjusted to this reaction. If, however, a sufficient amount of saponin be introduced into the acid solution, the reaction may be carried, by the gradual addition of alkali, over the isoelectric point without the occurrence of any precipitation whatever. It might be thought at first sight that we have to do here merely with an instance of inhibition of precipitation or supersaturation such as is known to occur in simpler cases in the presence of saponin; that such a straightforward explanation fails to fit the facts is shown, however, by the effect of lower concentrations of saponin. The saponin possesses apparently the additional power of, so to speak, sharpening the isoelectric points of the proteins present. If a solution of insulin in dilute acetic acid containing about 1 % of saponin is treated gradually with ammonia, a definite turbidity is observed when the p_H is about 4·5; in the absence of saponin, turbidity occurs earlier and a gross flocculation is already apparent at this reaction. On keeping the saponin-containing solution, a flocculent precipitate separates in the course of about 30 minutes, and may be removed on the centrifuge, when it is found to represent about 30 % of the weight of the starting material but only 15–20 % of the activity. Whilst continued addition of ammonia to the solution of insulin in dilute acid in the absence of saponin causes steadily increasing precipitation up to about p_H 5·0, further addition of alkali to the saponin-containing solution after removal of the precipitate at p_H 4·5 causes no visible

change until the reaction reaches p_H 5·6, at which point an opalescence develops; if the addition of alkali be interrupted and the solution kept overnight a precipitate forms which, in a satisfactory experiment, is already partly crystalline. A second similar isoelectric precipitation from more concentrated solution in presence of saponin results in a product which is almost entirely free from amorphous material, although not yet of very definite form. The precipitate may now, however, be successfully recrystallised by isoelectric precipitation from a phosphate buffer solution without the aid of saponin, and, by the selection of appropriate conditions of temperature and concentration, may be obtained in remarkably large and well-defined crystals.

Given, therefore, a suitable saponin we possess, in the technique summarised in the last paragraph, a simple and rapid means for the preparation of crystalline material from a commercial insulin powder of the unitage indicated. There are many factors, however, which render the situation less simple than is indicated by the above brief description. The difficulties of standardising any method requiring the delicate adjustment of physicochemical conditions, which involves the use of saponin, are obvious. Commercial saponins are crude substances of varying origin and variable properties; from the commercial point of view the value of a saponin is determined solely by its frothing capacity, and we were soon able to show that this property by itself fails to give an indication of the efficiency of a saponin for our purpose. We are indeed quite unable to offer any rational explanation of the mechanism of the action of saponin in promoting the crystallisation. That it is not, entirely at any rate, a question of the lowering of surface tension is indicated by the failure of substances such as the higher alcohols, which lower surface tension to a still greater extent, to achieve the same object, at least with a similar efficiency. Part of the effect is undoubtedly to be ascribed to the power of saponins to inhibit precipitation in general, and in so far as this is concerned, there is some indication that a high frothing capacity on the part of a saponin implies also a high capacity for promoting supersaturation. A not less important factor, however, is the apparent ability of certain saponins to effect the sharpening of the isoelectric points of the mixture of proteins which constitute commercial insulin, with the result that it becomes possible to precipitate the proteins individually at fairly widely differing and clearly defined points instead of throwing down the mixture *en masse* over a more extended range of p_H. It is evident that from the preparative standpoint these two properties of the saponin are to some extent mutually antagonistic. The more saponin used the greater is the degree of supersaturation induced and hence the lower is the yield of crystals; on the other hand, the crystallisation fails entirely to occur if enough saponin be not used to effect the separation of the isoelectric points (if we may so designate the phenomenon discussed above) in a well-defined manner. By a fortunate coincidence the first sample of saponin which we tried possessed eminently satisfactory properties in both respects. The next sample, although ostensibly

of similar quality, was almost entirely useless for our purpose; various subsequent samples of saponin have exhibited differing degrees of efficiency, as determined by the number of precipitations necessary for the purification and by the yield of crystalline product obtained. The cruder samples contained considerable amounts of calcium sulphate and traces of heavy metals; with such samples, as might be expected, immediate precipitation of part of the insulin occurred even in fairly acid solutions, and an unduly large amount of material was therefore removed with the first acid precipitate at p_H 4·5. Apart from the elimination of such gross impurities, however, it was difficult to detect any distinguishing feature by which a saponin might be recognised as being suitable for the purpose in hand. In the search for such a criterion we investigated the haemolytic power of the various saponins employed, for help in which part of the work we are indebted to Dr H. D. Wright; as will be seen from the results recorded below there does appear to exist at least a rough parallelism between the haemolytic indices of the different saponins and their efficiencies in promoting the crystallisation; as an approximation it may be said that, as far as our experience extends, a saponin with a haemolytic index of less than 1 : 8000 is unlikely to be of much use for the purpose.

Apart from the variability of the different saponins themselves, a further complication is introduced by the effect of surface. The method was first worked out for 20 mg. samples of the crude insulin powder, and we were for a long time troubled by our inability to extend the process to larger amounts. Thinking that the difficulty might be due to the importance of preserving correct time-relationships between the different stages of the manipulation, we performed many experiments on the adjustment of these relationships, all however to no purpose. We were then reminded of the fact that when a solution of saponin is introduced into a glass vessel a gradual concentration of the saponin occurs at the liquid-glass interface, and this was apparently the key of the situation. In the earlier small-scale experiments mentioned above the solution was always transferred, at the crystallising point, to 15 cc. centrifuge cups in which the liquid-glass interface was about 25 cm.^{2} for a total volume of 9 cc.; in the larger experiments involving greater volumes of liquid, the final solution was left in a beaker, or transferred to larger centrifuge cups of a different shape; in either case the ratio of liquid-glass interfacial area to volume of solution was significantly lower than that in the small experiments; in the case of the beaker the surface was only 45 cm.^{2} for a volume of 45 cc. Such larger experiments led to no good result, and it was only when, after many variations, we restored this ratio to its original value by dividing the final solution, as soon as it was adjusted to the correct p_H over a large number of the 15 cc. cups, that we were successful in achieving the crystallisation of larger amounts. We were subsequently able to bring about the same result by leaving the solution in the beaker and introducing the requisite number of glass rods to bring the interfacial area to the same proportionate amount as in the small tubes. It may be noted here that it is

in the first separation from the crude insulin powder that the surface relationship is of paramount importance; once the precipitate has been obtained even partly crystalline this factor becomes of less significance. In all probability there exists an optimum relationship between the concentration of saponin employed and the surface-volume ratio which we have just discussed, and the particular suitability of the 15 cc. centrifuge cups for our purpose is decided by the concentrations of saponin which we have arbitrarily selected. Much further experimental work, however, will be required to clear up this point, and, for the present, we have contented ourselves with the preservation of the correct relationship by the simple expedient indicated, since we have not attempted to extend the method to larger quantities than 0·5 g. of the crude powder. It is hoped in the near future to carry out further experiments with the object of defining the optimum conditions more exactly. In order to make the method, at least for small quantities, readily reproducible, we have worked out the details for the crystallisation using pure digitonin instead of a commercial saponin; the conditions for the use of ordinary saponins can at present only be described in somewhat general terms, the exact details having to be worked out for each sample.

Perhaps the least satisfactory feature of the whole method is the poorness of the yield. With the different saponins of which we have had experience this has varied from 5 to 15 % of the crude powder, which, considering the relatively small access in activity on crystallisation, evidently leaves much to be desired; we have, however, done little or nothing towards working up residues, and it is not unlikely that a systematic application to this task would effect a considerable increase in the total yield obtainable.

It will be apparent from what has been said that a large amount of experimental work remains to be done in order to realise to the full the possibilities of the method which we describe. Nevertheless we are publishing our results at the present stage, partly because our immediate association in the work can unfortunately not be continued, but also because we feel that, even so far as our experiments have gone, we have some evidence to offer bearing on the question of the identity or non-identity of the crystalline compound with insulin.

It may be said at once that the strongest evidence on the last-mentioned point is to be found in the results of the physiological assay contained in Part II of this paper. The impressive consistency of the results reported by independent workers for the activity of four different samples of crystals, two obtained by Abel's method, and two by our own, must, we think, be accepted as a powerful argument in favour of the identity of the compound with insulin. In addition to the results recorded in Part II assays were performed by the mouse method on samples of crystals prepared from the B.D.H. insulin by the use of saponin and from the insulin of Messrs Burroughs Wellcome by the aid of digitonin. In both cases the product showed an activity of 25 international units per mg.

To obtain evidence as impressive from the chemical side is a difficult matter, but there is no doubt that the results of our experiments point in the same direction. The crystals themselves exhibit a clearly defined structure, which is well depicted in the photograph (Plate I) (kindly taken for us by Mr J. E. Barnard); all preparations present an appearance of absolute uniformity, not only to our eyes, but to those of a crystallographer[1]; the possibility that they may represent a mixed crystal containing insulin as one component seems therefore remote. There remains the more likely suggestion that they may owe their physiological activity to the adsorption of a trace of a highly active substance, and this criticism is the more difficult to meet, since the crystals can only be obtained under one set of physico-chemical conditions, namely by isoelectric precipitation. We think, however, that the success of the saponin method does afford some evidence against the adsorption theory, since it is difficult to suppose that the conditions of adsorption would not be modified by the presence in the solution of so highly surface-active a substance as saponin, and yet the saponin method and that of Abel lead to products of identical activity. Further, we have been able to show, in confirmation of Abel, that the crystallisation may be effected from 50 % alcohol instead of from water without modification of the activity of the product. Negative evidence on the same point is provided by the failure of our attempts to recover any crystalline material from solutions of the crystals which had been subjected to the mildest chemical treatment consistent with destruction of the physiological activity.

The conception that insulin, with its high degree of specific physiological activity, should be a protein without any obvious distinguishing chemical property is admittedly not readily acceptable; still less acceptable to many minds will be the notion that the best commercial products, prepared by empirical methods, should represent almost pure insulin. The latter objection is, however, based on prejudice rather than on reason, and we suggest that both difficulties may be largely removed by a hypothesis which we wish tentatively to advance.

Let us suppose that we take thyroid gland, extract therefrom the iodothyreoglobulin, and, by some manipulation, succeed in obtaining the latter in the crystalline form. We should then have in our hands a crystalline protein exhibiting (although, owing to its large molecular weight, not in a very high degree) the specific physiological activity of the gland; we might, therefore, not unjustifiably, designate the product the pure active principle of the thyroid. In the light of our present knowledge we recognise that such a designation would be correct only in a limited sense, since the actual physiological activity is due, not to the protein as a whole, but to its specific constituent thyroxine. In a similarly limited sense we are inclined to regard the crystalline protein

[1] The crystals were kindly examined for us by Dr T. V. Barker of the Department of Mineralogy, University of Oxford, who expressed the opinion that the preparation had the appearance of "a definite substance with well-defined crystallophysical properties." The crystals were too small to permit of complete crystallographic measurements.

General field of crystals. × 120

Single crystal. × 340

Stereoscopic photograph of single crystal. × 340

PHOTOMICROGRAPHS OF INSULIN CRYSTALS

from the pancreas as being the true active principle of the gland in so far as the function of the latter in relation to carbohydrate metabolism is concerned. We feel that in this case, as in that of iodothyreoglobulin, the physiological activity is a property, not of the protein as a whole, but of some specific constituent or grouping contained within its molecule. The thyroid protein is differentiated from other proteins in an obvious manner by its iodine content, and the specific constituent, being a simple amino-acid, can be readily separated from the rest of the molecule; the insulin protein bears no such chemical earmark, unless it be the high content of sulphur. The analogy which we have drawn between the thyroid protein and the insulin protein must not be taken to indicate that we believe such a separation of the specific group to be necessarily practicable in the latter case. As a theoretical possibility, however. it seems worth bearing in mind.

Experimental.

The method of Abel. The first experiments consisted in attempts to apply the method described by Abel *et al.* [1927] to the preparation of crystalline material from the Toronto insulin powder assayed at 13 international units per mg. Although we followed the directions given by Abel with the greatest care, all our solutions being carefully checked, we met at the outset with a puzzling discrepancy in that we failed to obtain any precipitate on the addition of pyridine. In this respect the crude insulin was apparently behaving in a manner similar to that noted by Abel as characteristic of the crystalline material. We, therefore, proceeded with the addition of ammonia, using a 0·85 % solution as indicated by du Vigneaud *et al.* [1928][1]; after addition of the specified amount of ammonia we obtained a precipitate which was removed on the centrifuge, forming a somewhat gummy mass; nothing separated from the mother liquor on keeping the latter in the ice-chest, and, on testing, the solution was found to be considerably more acid than it should be according to Abel's description; further ammonia was added (3 cc. for every 0·1 g. of original insulin) to bring the reaction[2] of the solution to p_H 5·6. At this point an opalescence developed, and, on keeping the solution in the cold, a precipitate settled out, which was, however, amorphous. By repeatedly putting this precipitate through a similar process in continually diminishing volume (using always more ammonia than indicated by Abel) the material was eventually obtained in the crystalline condition as described by Abel, and was recrystallised in the absence of brucine either by the method given in his paper, or better from a phosphate buffer as described below. No explanation of the differences observed by us can at present be given. We mention our experience for the possible interest of other workers who may

[1] In the paper by Abel *et al.* [1927] the use of 0·65 % ammonia for the crystallisation is described; a subsequent paper by du Vigneaud *et al.* [1928] from Abel's laboratory gives 0·85 % as the concentration of this reagent, no reason for the change being given so far as we can ascertain.

[2] All p_H determinations mentioned in this paper were made colorimetrically.

be attempting to carry out this manipulation. The discrepancy was not due to excess of acid adherent to or combined with the original insulin, since this was neutralised at the outset; attempts at purification of the crude powder by the phenol treatment of Abel or by continuous extraction of an isoelectric suspension with butyl alcohol under diminished pressure failed to bring the behaviour of the insulin into line with that of the sample which Abel employed, although the butyl alcohol extraction, which removed a small amount of physiologically inert material (possibly of a lipoid character) did appear to improve the conditions for crystallisation to a small extent. At the best, however, the yields were so minute and the method so time-consuming that we soon passed on to the attempt to find an alternative procedure, particularly as we were anxious to break away entirely from the use of brucine—a substance which, in a case such as the present, has very obvious disadvantages.

Experiments with carbon dioxide. It was thought that the reaction of an alkaline buffer solution containing insulin might be adjusted so delicately by the gradual introduction of carbon dioxide that we might in this way achieve the necessary supersaturation at the isoelectric point, dispensing with the elaborate mixture of weak bases. With this object 0·1 g. of insulin was dissolved in 4 cc. of water together with 0·3 cc. of 0·1 N hydrochloric acid, and this solution was mixed with 10 cc. of a phosphate buffer at p_H 7·0 to which had been added 1·5 cc. of 0·1 N sodium hydroxide. A slow stream of carbon dioxide was passed into the solution through a capillary tube; after 3 minutes an opalescence was apparent. The addition of carbon dioxide was interrupted and the solution allowed to stand for some hours; the precipitate was separated on the centrifuge, and the mother liquor further treated with carbon dioxide; this caused almost immediate further precipitation, and by the time the reaction had reached about p_H 6·4 the greater part of the insulin had separated in the amorphous condition; no tendency to crystallise was observed at any stage, nor did any fraction obtained by precipitation with carbon dioxide appear to be more suitable for crystallisation by the brucine method than the original crude powder. These experiments were, therefore, not pursued further.

Saponin method. The observations on which this method is based have been discussed in the introductory part of this paper and need not be further referred to here; nor shall we do more than mention the very numerous experiments which have been made on the effect of variations in temperature and in the time-relations of different parts of the process, and on the optimum point at which to remove the first precipitate. In this section we propose to describe (*a*) the method by which we gauge the most suitable amount of saponin to employ, (*b*) the crystallisation with the aid of a good commercial saponin, (*c*) the method with the use of digitonin.

(*a*) *Determination of correct amount of saponin.* A 6 % solution of the saponin in distilled water is made. (This solution should be prepared freshly

each day owing to the rapidity with which it becomes infected.) A stock solution of insulin is then prepared by dissolving 0·1 g. of the commercial powder in 5 cc. of 10 % acetic acid and making the volume up to 25 cc. with distilled water. This stock solution is divided into five equal portions, and these are treated with 0·5, 0·75, 1·0, 1·5 and 2·0 cc. of the saponin solution respectively. The solutions are warmed to 35° and to each portion is added 1·6 cc. of 0·85 % ammonia. After standing for 30 minutes at 35° the precipitates which have formed are removed on the centrifuge, and the mother liquors are adjusted to p_H 5·6 by the addition of a further 1·7 cc. of the 0·85 % ammonia and transferred at once to 15 cc. centrifuge cups. It is important to adjust the reaction to p_H 5·6 as rapidly and as sharply as possible; the exact amount of ammonia necessary will vary slightly with different samples of insulin according to the amount of acid present. The tubes are allowed to stand, with occasional scratching with a glass rod, and are examined next day. With a good saponin the general result of such an experiment will be that in the tubes with the lower concentrations of saponin there is a large amount of amorphous precipitate whilst in those with the high concentrations the precipitate is mostly crystalline but small in amount; it is usually fairly easy to pick out that tube of the series in which conditions are best, *i.e.* where there is the largest amount of precipitation consistent with the absence of grossly amorphous material. It must be understood that, in speaking of crystalline material at this stage we do not mean to indicate that it already has a well-defined form; this is not the case, but with some experience it becomes quite easy, on examination under a high power of the microscope, to distinguish between material which, while not yet of definite form, will recrystallise satisfactorily, and the ragged grossly flocculent impurities which must be avoided. It is really only by experience that these differences can be recognised; a more precise verbal description is difficult to give. However, those tubes in which conditions are good are usually macroscopically distinguished by the separation of material on the sides of the vessel where it has been scratched with the glass rod. In the case of our best commercial saponin optimum conditions obtained in the tube to which 1·0 cc. of the saponin solution had been added; in this case, therefore, a typical experiment was as follows.

(*b*) *Crystallisation with commercial saponin.* To 5 cc. of the stock solution of insulin in 2 % acetic acid (representing 20 mg. of the crude powder) was added 1 cc. of 6 % saponin solution; the mixture was warmed to 35°, treated with 1·6 cc. of 0·85 % ammonia, and kept at 35° for 30 minutes; the precipitate was removed on the centrifuge and the mother liquor adjusted as rapidly and sharply as possible to p_H 5·6 by the addition of 1·7 cc. more of the ammonia, and transferred immediately to a 15 cc. centrifuge cup. At this point the solution was still clear or only very slightly opalescent; on standing and scratching with a glass rod a turbidity soon developed, and overnight a precipitate separated which was largely micro-crystalline in

character. Five such tubes were usually prepared simultaneously, or 100 mg. of the crude powder was worked up as a whole, the final solution being divided over 4 or 5 tubes; after separation was complete the precipitates were collected with the aid of the centrifuge and combined in solution in 2·5 cc. of 10 % acetic acid and 10 cc. of water; 2·5 cc. of the 6 % saponin were then added and the reaction adjusted immediately to p_H 5·6 by the addition of 8·25 cc. of 0·85 % ammonia. This time the precipitate was almost entirely microcrystalline, although not yet of definite form. A further crop of crystalline material could sometimes be obtained by saturating the mother liquor with butyl alcohol. The final crystallisation was effected as follows. The whole of the precipitate after the second separation in presence of saponin (representing 100 mg. of crude insulin) was dissolved in 4 cc. of water and 0·4 cc. of 0·1 N hydrochloric acid; the solution was poured with stirring into a mixture of 16 cc. of a phosphate buffer at p_H 7, prepared according to Clark [1923], to which had been added 0·8 cc. of 0·1 N sodium hydroxide; the resulting solution was warmed to about 55°, treated with 1·0 cc. of 0·1 N hydrochloric acid, which brought the reaction to p_H 5·6, and set aside. Within a few hours there separated a precipitate which was now wholly crystalline and exhibited the characteristic form. The size of the crystals could be increased by one or two further separations from phosphate solution on the same lines. The best results in this last crystallisation were obtained if the solution, after adjustment to the isoelectric point, were left entirely undisturbed so that the material should separate as slowly as possible. The yield varied with the saponin and the crude insulin employed, amounting to 5–15 % of the starting material. A physiological assay in one case indicated that 50–60 % of the activity remained in the combined mother liquors, 15–20 % being lost in the first acid precipitate, which however amounted to 30 % of the weight of the original insulin; this partition probably varies somewhat with different saponins and different preparations of insulin. We have, up to the present, made no attempt to subject the material recovered from the mother liquor to crystallisation; that it would almost certainly be possible to do so with more or less success is indicated, however, by an experiment in which we were able to obtain crystals from a crude powder of as low an activity as 8 international units per mg.

We have had experience in all of nine samples of saponin of which the first was considerably the best; this was a sample of "white saponin" purchased from the British Drug Houses. The second sample, purchased under the same description, turned out to be practically useless; subsequently we tried a number of commercial samples, all of which were more or less useful for our purpose, although some of the cruder ones, containing as they did large amounts of inorganic matter, even including traces of heavy metals, caused an apparent denaturation of a part of the insulin. In the attempt to find some correlation between a measurable physical property of a saponin and its usefulness for our purpose we have compared in Table I the frothing

capacity and haemolytic power of the various saponins with their efficiency in promoting crystallisation. In this table the frothing number is given as that concentration which on shaking for 15 seconds in a tube of 16 mm. diameter produces 1 cm. of froth measured after 15 minutes. The haemolytic power is expressed as the minimum concentration necessary to produce complete haemolysis of a 5 % suspension of rabbit's washed red blood-corpuscles in 0·9 % sodium chloride during 2 hours at 37°. (For these determinations we are indebted to Dr H. D. Wright.) Unfortunately the data are not available for the first and best of our saponins, since we had exhausted the supply of this material before running into the difficulties which persuaded us to make the comparison. The true haemolytic value for digitonin should probably be somewhat higher than that given, since part of the digitonin is precipitated, presumably by the cholesterol from the cells, and is thus removed from the solution. The general result of the comparison is to indicate that, from the point of view of efficiency in promoting the crystallisation, the haemolytic index of a saponin is of more significance than is its frothing power.

Table I.

Saponin	Frothing number	Haemolytic index	Crystallising efficiency
White No. 1	Not determined	Not determined	+ + +
,, ,, 2	1 : 25,000	1 : 4,000	Useless
,, ,, 3	1 : 25,000	1 : 8,000	+ +
A	1 : 20,000	1 : 32,000	+ +
B	1 : 16,000	1 : 32,000	+ +
C	1 : 15,000	1 : 32,000	+ +
D	1 : 15,000	1 : 32,000	+
E	1 : 9,000	1 : 16,000	+
F	1 : 10,000	1 : 32,000	+ +
Digitonin	1 : 30,000	1 : 8,000	+ +

Digitonin method. The insulin powder (0·1 g.) is dissolved in 5 cc. of 10 % acetic acid and the solution is made up to 25 cc. with water; 0·125 g. of digitonin dissolved in 2·5 cc. of water is now added, followed by 8 cc. of 0·85 % ammonia; after standing 30 minutes, the precipitate is removed on the centrifuge and the mother liquor adjusted to p_H 5·6 by the addition of 8·5 cc. of 0·85 % ammonia; the solution is then divided over four or five 15 cc. centrifuge cups and set aside; after standing for some hours (preferably overnight) the precipitate is separated on the centrifuge and subjected to a similar process, using half the quantities of all reagents; after the second separation the precipitate may be successfully crystallised from a phosphate solution exactly as described above. In the case of the digitonin, as in those of some of the commercial saponins, there may be little sign of actual crystallisation as the result of the first two precipitations, the visible improvement consisting rather in the elimination of the grossly flocculent amorphous material; so long as the removal of the latter is complete, however, the final crystallisation from the phosphate solution succeeds. The yield by the digitonin method has not been more than 10 % of the starting material.

Miscellaneous experiments.

(*a*) *Butyl alcohol.* The observation noted above that a further crop of crystalline material may be obtained from saponin-containing mother liquors, combined with the consideration of the part which must be played in the whole process by the lowering of the surface tension of the solution produced by saponin, led us to try some experiments on the effect of butyl alcohol alone. Crude insulin was, therefore, subjected to a process similar to the phosphate crystallisation described above except that 5 % of purified *iso*butyl alcohol was added to the alkaline solution immediately before adjustment to the isoelectric point. In this way we were indeed successful in obtaining some material which appeared partly crystalline, but attempts at recrystallisation did not lead to a satisfactory product. It was, moreover, noticed that part of the material had lost its acid-solubility as the result of the butyl alcohol treatment, *i.e.* was partly denatured, and these experiments were, therefore, not pursued further.

(*b*) *Bile salts.* In the search for a substitute for saponin of a more reproducible character than the latter, an experiment was made to see whether bile salts could be employed; it was found, as was anticipated, that they were useless in solutions of the acidity which we were employing.

(*c*) *Inactivation experiments.* In order to obtain some evidence bearing on the question of the identity of the crystalline material with insulin, particularly in relation to the adsorption theory discussed in the introductory part, attempts were made to recover crystals from solutions of the latter which had been subjected to the minimum treatment with alkali which was necessary to bring about more than 80 % inactivation. The crystals (20 mg.) were dissolved in an alkaline phosphate solution (40 cc.), the p_H being approximately 10·5; in order to produce the required degree of inactivation, this solution had to be heated at 70° for 30 minutes; after such treatment, on readjustment to p_H 5·6, only amorphous material separated, nor could any crystals be obtained by subjecting this material to the saponin treatment. The inactivation by alkali was apparently irreversible, *i.e.* no regeneration of activity or of potentially crystalline material was observed on making the reaction acid and keeping the solution.

Appearance and analysis of the crystals.

The preparations of crystalline material present always an appearance of absolute uniformity. Seen under a low power the most conspicuous feature is the hexagonal outline noted by Abel. Examined more closely under a high power the crystals present the appearance of cubes, or rhombohedra approximating to cubes, which are standing on one corner, thus giving rise to the hexagonal outline already mentioned. This description has naturally not the slightest crystallographic significance, and the crystals are too small for complete angular measurements to be made. They are weakly doubly-refracting and have a refractive index of approximately 1·58. The optical appearance of

the crystals is better depicted in the accompanying photographs (Plate I) than it can be described in words.

For analysis, some of the material was dried in a vacuum desiccator over sulphuric acid at the ordinary temperature and then allowed to come to equilibrium with the air; analysed in this condition it gave the following figures, which agree closely with those given by Abel for his air-dried substance[1].

4·861 mg.	gave	8·79 mg. CO_2, 2·9 mg. H_2O
4·960	,,	8·93 mg. CO_2, 3·08 mg. H_2O
2·945	,,	0·365 cc. N_2 at 21·5° and 767 mm.
3·028	,,	0·364 cc. N_2 at 22° and 767 mm.
11·200	,,	2·535 mg. $BaSO_4$

	C	H	N	S
Whence	49·61	6·81	14·49	3·11
	49·11	6·95	14·03	—

PART II.

In Part I of this paper is described the preparation from insulin of crystals, indistinguishable from those obtained by Abel and his colleagues, by the aid of a simpler method than that which they have used. Preliminary trials showed that these crystals possessed the specific activity of insulin in a high degree. It appeared to be important to have this physiological activity measured as accurately as the available methods allow. Two questions concerning the activity of such insulin crystals seemed to us to require more definite answers than they have hitherto received.

1. Do the crystals, apparently pure and identical, obtained by different procedures, show a uniform activity, or an activity which varies from batch to batch? A precise answer to this question would have an obvious bearing on the claim of the crystals to be regarded as insulin itself in pure form. A wide variation in activity from batch to batch would be inconsistent with such an identification; uniform activity, on the other hand, in batches prepared by different methods from different samples of the crude insulin, while not affording conclusive proof that the crystals are pure insulin, would be difficult to reconcile with the view that they consist of a non-specific, inert material, on which the insulin is merely adsorbed.

2. If the crystals show uniform activity, what is the degree of that activity, expressed in the commonly accepted units?

In spite of the large amount of work on the subject during the past two years, the information on these two points, obtainable from the literature, is curiously incomplete and unsatisfactory. In his first paper on the subject Abel [1926] did not attempt to assign a definite unitage to the crystals, merely indicating that 1/100 mg. in one experiment, and 1/125 in another, had sufficed to reduce the blood-sugar of certain rabbits to the traditional

[1] Analyses performed by Dr Ing. A. Schoeller, Berlin-Schmargendorf, Tolzerstrasse 19.

"convulsant level" of 45 mg. per 100 cc. and even to produce actual convulsions. In a later paper, published with Geiling, Rouiller, Bell and Wintersteiner [1927], he assigned a value of 90 units per mg. to a sample of the crystals. More recently du Vigneaud, Geiling and Eddy [1928] have recorded a value of 54 units per mg., and Jensen and Geiling [1928] one of 45 units per mg., for different samples of the crystalline product[1].

Taken at their face value, these estimates do not give an impression of uniform activity in the different samples; nor, with estimates spread over so wide a range, is it possible to select one as representing the true and characteristic activity of the crystals; though the fact that actual convulsions are recorded in certain rabbits, receiving as little as 0·01 mg., suggests an activity higher than that of the purest insulin obtained by ordinary methods. On none of these points, however, can we regard the evidence of these measurements as satisfactory or convincing, since they were all obtained by the old method of direct calculation from the degrees of hypoglycaemia produced in unstandardised rabbits. It is not possible to attach a precise significance to unit values which have not been measured by comparison with, and expressed in terms of the International Standard, which since 1925 has afforded the basis for the unit by international agreement and general use. Such measurements of the activity of the insulin crystals, in relation to the International Standard, have been published by Freudenberg and Dirscherl [1928], who obtained the values of 23 and 26 units by different comparative methods. The difference between these two figures is not greater than the error of any method yet available; but the potency which they indicate is only about one-half of those more recently published from Abel's laboratory.

Plan of the investigation.

Four lots of the crystals were selected, two prepared by Abel's method, and two by the new method described in Part I. Taking them in the order in which they were examined they were as follows.

A. Crystals obtained by Harington and Scott, using Abel's method, from Toronto insulin having a unit value of 13–15 units per mg.

Test solution A made by dissolving 10·2 mg. in 50 cc., *i.e.* 0·204 mg. per cc.

B. Crystals from the same Toronto insulin, prepared by Harington and Scott's "saponin" method.

Test solution B contained 11·8 mg. in 50 cc., *i.e.* 0·236 mg. per cc.

C. Prepared from the same raw material, and by the same method as B, but on a different occasion.

Test solution C contained 10·2 mg. in 50 cc., *i.e.* 0·204 mg. per cc.

[1] In a recent private communication to Dr Dale, Prof. Abel states that his colleagues are now obtaining values of 3 to 3½ times the activity of the International Standard (*i.e.* 24–28 units) for different samples of the crystals assayed in comparison with that Standard.

D. A mixture of two small lots of crystals kindly furnished by Prof. Abel, and prepared by his methods in his own laboratory.

Test solution D contained 8·2 mg. in 50 cc., *i.e.* 0·164 mg. per cc.

It was decided that each should be assayed independently by the four authors of this part, each of whom has long familiarity with the standardisation of insulin for practical use. Two different types of method were used. Scott and Trevan used the essentially statistical method, based on the relative frequencies of the appearance of hypoglycaemic convulsions in a large number of mice injected with a dose of the preparation under test, and in an equal number of similar mice injected at the same time with a control dose of the standard preparation, and observed under identical conditions. The method was independently elaborated, and with some differences of detail, by Trevan and Boock [1926], and by Krogh [1926]. Both Marks and Culhane used the method based by Marks [1925, 1926] on parallel observations of the degree of hypoglycaemia produced, by a dose of the preparation under test and by a dose of the standard preparation, in the same series of rabbits on different occasions; changes in the sensitiveness of the whole series being eliminated by injecting half the number with each preparation on each occasion, and reversing the groups on the two days of the test. Variations in the details of the application of these tests are mentioned below.

So that no question should arise of differences in the weighing out or solution of the preparations, a sufficient quantity of solution for the tests of all four investigations was prepared by Dr Harington from each of the batches of crystals. Physiological saline, acidulated with HCl to p_H 2·5, was used for the solution of the crystals, and the solutions were not made up to identical strengths of the crystalline material. In each case a quantity in the neighbourhood of 10 mg., but varying in the four cases from 8·2 to 11·8 mg., was dissolved and made up to 50 cc.

A common stock solution of the International Standard Insulin was similarly prepared by Marks, who weighed out and dissolved the dry powder in distilled water, making a solution of acidity about p_H 3·5 which contained 2·5 mg. of the standard preparation, and therefore had an activity of 20 units per cc. For each test each of us received a sufficient quantity of the standard solution, containing 20 units per cc., and of the solution of one of the crystalline preparations, of which the concentration as well as the activity was unknown to him. It was understood, however, that, in order to save time and animals, the solutions of A, B, C and D would not differ widely in concentration.

Each of us completed the determinations on any one of the preparations, A, B, C and D, without consultation with the others. In each case we handed our estimates independently to Dr Harington or Dr Dale, and they were not divulged or submitted to us for comparison until all four had been deposited.

Estimates by D. A. Scott.

The experiments were carried out at the National Institute for Medical Research, with the equipment there available. The mouse method, as advocated by Krogh and by Trevan and Boock, was used. The details followed were mostly those given by Trevan and Boock [1926]. The thermostat bath, in use at the National Institute to maintain the temperature in the mouse-boxes during the experiment, follows the general lines of Trevan's description, but has a motor-driven stirrer, an electrically-controlled thermostat, and a convenient weighting of the boxes.

The method has been for some time in use in the Connaught Laboratories, University of Toronto, with the following departures in detail from Trevan and Boock's description.

1. Mice showing convulsions in the course of a test are restored by injection of glucose, and after a week the whole stock are found to be as suitable for a further test as a fresh stock of mice.

Krogh's experience and practice are here confirmed and followed.

2. The volume of solution injected into each mouse is 0·25 cc. According to our experience a larger injection volume entails danger of leakage. Since the mice used in any experiment are all of about the same weight, not varying more than 1 g. in either direction from the mean, the mean weight can be accepted for all, and the required dose per g. of standard or test solution made up so as to occupy 0·25 cc. per mouse.

3. Observation was completed in 1½ hours, in place of the 2 hours indicated by Trevan. Since about 90 % of the mice which show convulsions do so within the first hour, the time of observation could probably be made even shorter.

The mice used in these tests were all of weights between 19 and 21 g. All but a few were bred at the National Institute, and were from 2½ to 3 months old at the time of their first use for these tests. The diet of all consisted of white English oats and stale brown bread, the latter being moistened with water. The mice to be used in an experiment were transferred to an empty cage and deprived of food at 5 p.m. on the previous afternoon. The injections were begun at 10.30 a.m. Injections were subcutaneous, and were made with a convenient precision-syringe of 0·25 cc. capacity. Each mouse, after injection, was immediately transferred to its appropriate box in the thermostat bath, which was kept throughout the experiment at 37·5–38°. The mice were kept as quiet as possible, and the number showing actual convulsions, or equivalent symptoms of collapse, was recorded. Trevan's test, depending on the inability of a mouse to right itself immediately, when placed on its back, was found useful in the recognition of symptoms "equivalent" to convulsions. Such mice, when the reaction was established, were removed and given a hypodermic injection of 0·2 cc. of 20 % glucose. At the end of 1½ hours the score was regarded as completed, and the mice which had not

shown definite "convulsions" or "symptoms" were removed, and food was given to the whole stock.

The determination of the unknown dose of the solution under test, from the relation between the proportions of convulsions produced by it and by the known dose of the standard, was made from the curve published by Trevan and Boock. After a preliminary orientating test, giving the approximate unit value of the unknown solution, an attempt was made in subsequent tests to give this and the standard in approximately equivalent dosage, and in each case to produce a convulsion-rate in the neighbourhood of 50 %. From the actual rates observed the true dose can then be read off the curve with a relatively small error.

The protocols are given at the end of the next section. From these it is seen that the figures obtained range from 24·2 units per mg. for D to 25·35 units for C.

Estimates by J. W. Trevan.

The tests were carried out by the mouse method as originally described by Trevan and Boock [1926], with the modification that the weights of the mice used were matched for the two solutions under test. Mice of 15–18 g. were used for one set of tests, mice from 18 to 25 g. (a very few being over 25) in another. None was used below 15 g. We have taken this precaution for some time.

The protocols are given at the end of this section. The figures in the last column of each table are calculated from the standard curve given in the original description of the method. The values for tests 1 and 2 on A are calculated from convulsion rates which are too low for much reliance to be placed on the calculated potency, although the results do agree with the later ones. However, the deduction can be made that the characteristic for insulin A is not significantly different from that of the International Standard given in the paper mentioned above.

One test (No. 5) on A was made, in which only low dilutions were used, and minute doses both of the standard and of A were injected by means of micrometer syringes. This was done to check whether in high dilutions the purer insulin was destroyed by surface action or shaking. No significant difference was found.

From the protocols, it is seen that the average figures vary between 22·8 units per mg. for C and 25·8 units for B.

Discussion of mouse test results.

The results of the mouse test by both workers are collected and compared in Table I. The degree of uncertainty of the final results is represented by the columns headed "range of possible values." These figures are calculated from the shape of the mouse insulin characteristic already published, and from the number of mice used, by the use of the formula given by Trevan

Table I. *Combined results and limits of error of mouse tests.*

	Scott		Trevan		
	Mean value (Units per mg.)	Calculated possible range	Mean value (Units per mg.)	Calculated possible range	Combined result
A	24·9	21·75–28·60	24·2	23·4–26·4	24·4
B	25·0	21·85–28·70	25·8	23·6–28·2	25·6
C	25·35	21·15–29·0	22·8	20·9–25·0	23·6
D	24·2	21·65–27·5	24·4	21·8–27·3	24·3
	Mean 24·8		Mean 24·3		Mean 24·5 (23·6–25·4)

[1929], assuming that the average convulsion rate is 50 %. This gives the error corresponding to approximately twice the standard deviation of the convulsion rate, and represents the range inside which the true value may be expected to fall with a probability of approximately 0·95. The discrepancy from the total average value of any single set of tests is not greater than might be expected to occur by errors of random sampling of mice. The most discrepant result is that of Trevan for C. The difference of this from the corresponding result by Scott on the same solution is, however, only 1·26 times its standard deviation. It is interesting that the individual values obtained for C by Trevan were much more concordant than those obtained

Protocols of mouse tests carried out by D. A. Scott.

	International standard 20 units per cc.			Solution of crystalline insulin			
Test No.	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Units per cc.
				Sample A.			
1	1/300	0·25 cc.	11/24	1/75	0·25 cc.	13/24	5·42
2	,,	,,	19/24	1/90	,,	15/24	5·05
3	,,	,,	18/24	1/75	,,	16/24	4·56
4	,,	,,	13/24	1/75	,,	15/24	5·42
						Mean	5·09
				Sample B.			
1	1/300	0·25 cc.	15/24	1/75	0·25 cc.	20/24	6·30
2	,,	,,	17/24	,,	,,	18/24	5·18
3	,,	,,	18/24	,,	,,	21/24	5·88
4	,,	,,	15/24	,,	,,	20/24	6·30
						Mean	5·9
				Sample C.			
1	1/400	0·25 cc.	20/24	1/100	0·25 cc.	19/24	4·72
2	,,	,,	16/24	,,	,,	18/24	5·45
3	,,	,,	17/24	,,	,,	20/24	5·70
4	,,	,,	16/24	,,	,,	15/24	4·80
						Mean	5·17
				Sample D.			
1	1/400	0·25 cc.	11/24	1/80	0·25 cc.	11/24	4·0
2	,,	,,	13/24	,,	,,	10/22	3·53
3	,,	,,	12/24	,,	,,	15/24	4·49
4	,,	,,	13/24	,,	,,	17/26	4·87
5	1/350	,,	15/24	1/70	,,	12/24	3·55
6	,,	,,	12/24	,,	,,	11/24	3·84
7	,,	,,	13/24	,,	,,	11/24	3·69
						Mean	3·97

for the other solutions, and this furnishes a good example of the uncertainty of relying on mere concordance of a limited number of observations as a guarantee of the accuracy of a result. The average result of the potency of all samples by the mouse test is 24·5 with a range of 23·6 to 25·4, the latter being worked out from the total number of mice used, namely, 2652.

Protocols of mouse tests carried out by J. W. Trevan.

	International standard 20 units per cc.			Solution of crystalline insulin			
Test No.	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Units per cc.
				Sample A.			
1	1/1000	0·5 cc.	25/30	1/1000	0·5 cc.	1/30	5·38
	,,	,,	19/30	,,	,,	0/30	
	,,	,,	21/29	,,	,,	0/29	
	,,	,,	19/30	,,	,,	1/30	
2	,,	,,	21/30	1/500	,,	2/30	4·37
	,,	,,	17/30	,,	,,	0/30	
	,,	,,	21/30	,,	,,	4/30	
3	1/1333	,,	19/30	,,	,,	15/30	6·61
4	,,	,,	18/30	1/400	,,	12/30	5·02
5	,,	,,	13/30	,,	,,	7/30	4·69
6	,,	,,	25/30	,,	,,	15/30	4·26
7	1/16	0·008 cc.	21/30	,,	0·008 cc.	24/30	5·75
8	1/100	0·5 cc.	24/30	1/250	0·5 cc.	16/30	3·82
9	,,	,,	18/30	,,	,,	10/30	4·68
10	,,	,	15/30	,,	,,	10/30	4·17
11	,,	,,	16/30	,,	,,	18/30	5·33
				Mean omitting tests 1 and 2			4·92
				Mean including tests 1 and 2			4·92
				Sample B.			
1	1/1000	0·5 cc.	20/30	1/250	0·5 cc.	18/30	4·67
2	,,	,,	17/30	,,	,,	21/30	5·70
3	,,	,,	19/30	,,	,,	24/30	6·00
4	,,	,,	23/30	,,	,,	25/30	5·40
5	,,	,,	12/30	,,	,,	25/30	7·85
6	,,	,,	14/30	,,	,,	17/30	5·51
7	,,	,,	12/30	,,	,,	23/30	7·22
8	,,	,,	21/30	,,	,,	26/30	6·15
						Mean	6·08
				Sample C.			
1	1/1000	0·5 cc.	21/30	1/250	0·5 cc.	15/30	4·11
2	,,	,,	17/30	,,	,,	17/30	5·00
3	,,	,,	22/30	,,	,,	18/30	4·46
4	,,	,,	22/30	,,	,,	20/30	4·65
5	,,	,,	20/30	,,	,,	19/30	4·83
6	,,	,,	18/30	,,	,,	17/30	4·83
7	,,	,,	24/30	,,	,,	22/30	4·61
						Mean	4·64
				Sample D.			
1	1/1000	0·5 cc.	13/30	1/250	0·5 cc.	6/30	3·69
2	,,	,,	10/27	,,	,,	8/27	4·61
3	,,	,,	16/30	,,	,,	8/30	3·74
4	,,	,,	10/30	1/200	,,	11/30	4·12
5	,,	,,	22/30	,,	,,	16/30	3·69
						Mean	3·97

Estimates by H. P. Marks.

For estimating the potency of the various samples of crystalline insulin, the rabbit cross-over method was used, on the lines laid down in the original communications on this test [Marks, 1925, 1926], but with the following modifications, suggested by later experience.

(1) *Weight of rabbits and dosage of insulin.* Culhane [1928] states that the test gives more consistent results when the two groups of rabbits are so chosen that, for each animal in one group, there is a corresponding animal of approximately the same weight in the other group. This procedure was therefore adopted, and it has the added advantage that the same dose of insulin may be given to all the rabbits regardless of body-weight, without disturbing the balance of the two groups.

With one or two exceptions, rabbits weighing between 2 and 3 kg. were used, and the dose of standard insulin throughout was 1 unit per rabbit, injected subcutaneously.

The standard solution was diluted ten times for use, so that the volume injected was in every case 0·5 cc. Either 10 or 12 rabbits were used for a test.

The danger of choosing a dose falling on the upper, flat portion of the dosage-response curve has already been pointed out [Marks, 1926]. In order to avoid obtaining a false match between sample and standard, the dose of sample to be compared with 1 unit of the standard was accordingly varied from one test to another, and, in all cases, this was reflected as a change in relative response.

(2) *Treatment of blood samples.* The simplified method of dealing with the samples of blood already suggested in the communications cited above was adopted. It consists in combining the five hourly blood samples taken from the same rabbit after insulin. The blood-sugar value determined on this composite sample, when subtracted from the initial value, then gives the average fall in blood-sugar during the five hours.

According to Sahyun and Blatherwick [1928], if a rabbit is given a certain dose of insulin after a fasting period of 24 hours, and the fasting continued and the same dose of insulin given again after a further 24 hours, the hypoglycaemic response to each dose is approximately the same. This observation seemed to offer such an attractive possibility of shortening the time required for the cross-test that the matter was immediately investigated, and it was found that to the same dose of insulin, repeated on the second day, the response was slightly greater than on the first day. Although there were occasional exceptions on the part of individual rabbits the results were certainly no less consistent than if the rabbits had been fed after the first test, and prepared again for a second test two or three days later.

In all tests except the first test on A, and the final test on D, the following procedure was therefore adopted.

Food was withdrawn from the rabbits in the afternoon, and on the following day the first part of the cross-test was performed as usual. Instead of feeding the rabbits after the test, fasting was continued and the second part of the test was carried through on the next day, after which the rabbits were fed. Rabbits used in this way regularly once a week (*i.e.* one complete test per week) suffered no ill effects, and increased in weight at the usual rate.

The results of the tests are collected in Table II.

Table II.

Sample	Dose of solution in cc. per rabbit	Estimated units in this dose	Units per mg. of crystals
A	0·167	0·88	26·0
	0·2	1·00	24·4
			Average 25·2
B	0·2	1·07	22·6
	0·187	0·92	20·8
			Average 21·7
C	0·2	0·90	22·0
	0·22	0·96	21·4
			Average 21·7
D	0·2	0·89	27·4
	0·225	0·99	26·9
	0·25	1·01	24·6
			Average 26·3

The estimated activity of the dose was in all cases calculated by reference to the dosage-response curve already published.

Estimates by K. Culhane[1].

The determination of the potency of the four solutions was carried out by the cross-over method of Marks [1926] with no modifications except that certain precautions were taken with regard to the selection of suitable animals and the distribution of their weights [Culhane, 1928].

(1) *Choice of rabbits and dosage of insulin.*

The rabbits were selected from young animals which had been used previously for testing a few of our purest samples and found to give consistent results. These were divided into groups of approximately equal weights, the weights of all falling between 1600 and 2400 g. The standard was diluted so as to contain 2 units per cc. and the samples were also diluted to a volume expected to contain the same amount per cc., all doses, both of the sample and of the standard being adjusted to the weight of individual rabbits: the dose of the standard was always 0·5 unit per kg. of body weight.

In almost all of the tests 20 rabbits were used, but on rare occasions one or two were excluded from the result. This was only done when the rabbit

[1] It is a pleasure to acknowledge my indebtedness to Dr S. W. F. Underhill, Director of the Insulin A.B. Physiological Laboratories, for his valuable help and criticism during this investigation.

showed signs of illness during the course of the test or went into convulsions on one of the doses or gave a percentage reduction greater than 50 %, since such an animal is considered too sensitive for quantitative work.

(2) *Preliminary starvation period.*

All animals were starved for 22 hours preceding the test and fed immediately after the fifth hour after the injection. The second half of the test was carried out three or four days later after the same period of starvation.

(3) *Treatment of blood samples.*

To enable us to use so large a number of animals in one test the five hourly blood samples for each rabbit after injection were mixed and a practical average thus determined.

(4) *Calculation of results.*

Experience has shown that results which differ greatly from 100 % are often inaccurate, and, whilst there appears to be a general tendency for the error to cause them to approach nearer to 100 %, we have found it more useful to disregard such tests, except as an indication of a more suitable dose for the next, than to include their results in an average after applying the correction suggested by Marks [1925]. Hence, the result of the final test gives our conclusion for the potency of any solution. In one case only have we departed from this procedure, namely that of the last sample, of which only a small quantity was available: as all the results come within 86 and 89 % there seemed no reason for regarding one test as more accurate than another and the three have therefore been averaged, allowance being made for the number of rabbits used in each.

(5) *Results of the tests.*

The tests are summarised in Table III.

Table III.

Sample	Dose of solution in cc. per kg.	Result as percentage of standard	Units per cc.	Units per mg. of crystals
A	(1) 0·083	79	4·7	
	(2) 0·106	87	4·1	20·1
	(3) 0·125	102	4·1	
B	(1) 0·125	107	4·3	18·2
C	(1) 0·119	104	4·4	21·6
D	(1) 0·119	89	3·7	
	(2) 0·135	86	3·2	20·7
	(3) 0·135	89	3·3	

SUMMARY OF RABBIT TEST RESULTS.

Since not more than three rabbit tests were performed by either worker on any one sample, it is impossible to form any opinion as to the comparative degree of accuracy of the two sets of figures. It is readily apparent, however, that Culhane's figures show less variation in activity between the different samples than do those of Marks. If all the tests carried out by each worker

respectively are considered on the assumption that all the samples have the same real activity, we find that the average of all Culhane's results (excluding test A 1, as being below 80 %) is 20·3 with a standard deviation of 0·5 for the mean, while the corresponding figures for Marks's tests are 24·0 and 0·75 respectively. Put in another way we may say that the probability is 0·95 that the true mean value lies between 19·3 and 21·3, according to Culhane's figures, or between 22·5 and 25·5 according to Marks's figures. Taken as a whole, all the rabbit tests give an average figure of 22·3 with a standard deviation of 0·7 for the mean, *i.e.* the probability is 0·95 that the true value lies between 20·9 and 23·7.

Conclusions and discussion of combined results.

If we review the results obtained by the different investigators, as presented in the preceding sections, it becomes clear at once that there is no wide divergence between the values obtained by any one of us for the four different samples. The largest such difference is that between B or C and D obtained by Marks, but as this is not supported by the other investigators, no significance can be attached to it.

A comparison of Culhane's results with those obtained by Scott and Trevan would suggest that the rabbit test gives a consistently lower value for the crystals than the mouse test, but against this must be set the relatively close agreement between Marks's average of 23·7 for all four samples, and the average of 24·5 for all the mouse tests. It would indeed not be surprising if the rabbit test and mouse test should give somewhat different results when comparing insulin preparations of different degrees of purity, since the physiological reaction forming the basis of measurement is different in the two cases. Even if we assume that the crystals represent pure insulin, we must suppose, from our own data, that the standard preparation contains approximately two-thirds of its weight of other substances, of which some may not be physiologically inert. If the resultant effect of these should be to inhibit, even slightly, the convulsant action on mice, while disturbing less the average hypoglycaemic effect produced in a rabbit over a period of hours, the result would be to show a higher unit value for the crystals in the mouse test than in the rabbit test. Again, differences in the rate of absorption of the injected insulin, if such occurred, might account for the discrepancies observed. We can only say that the course of the hypoglycaemia, which was studied in some of the rabbit tests, gives no clear indication of any difference in this respect between the crystalline insulin and the International Standard. Realising these inevitable uncertainties of the comparative methods of biological measurement, we consider that the fairest procedure is to average the results for the whole series. In Table IV, therefore, we have averaged the results of each investigator for all four samples, and of all four investigators for each sample.

Table IV.

	A	B	C	D	Average
Marks	25·2	21·7	21·7	26·3	23·7
Scott	24·9	25·0	25·3	24·2	24·8
Trevan	24·2	25·8	22·8	24·4	24·3
Culhane	20·1	18·2	21·6	20·7	20·2
Average	23·6	22·7	22·9	23·9	

Looking at the results as a whole, we think that the two questions which we propounded at the outset may fairly be answered as follows.

1. Different batches of the crystals, prepared by different methods, have no differences of activity which the available methods of assay can detect.

2. The activity shown by all the four samples assayed in comparison with the International Standard, and based on the average of the values obtained by the four investigators for each sample, may be assessed at 23·3, with a standard deviation for the mean of $\pm$ 0·6.

This latter result is in close conformity with the values of 23 and 26 units obtained by Freudenberg and Dirscherl [1928] for the particular sample of the crystals which they tested. We can state with some confidence that a sample of such crystals as we have tested, if adopted in the future as a stable standard of reference, might be assumed to possess three times the activity of the present standard, *i.e.* 24 units per mg., without any significant disturbance of the biological unit, as defined in terms of the current International Standard.

The uniform activity of the four batches of crystals seems to us to indicate that their substance has a closer relation to the specific insulin activity than that of an inert adsorbent of an intensely active contaminant. On the other hand, the activity of the crystals is little, if at all, different from that of the most active amorphous insulin, obtainable by pushing to the limit the fractionations used in ordinary manufacture. We are familiar with preparations, commercially available in the ordinary way, in which the unit value per mg. is always in the neighbourhood of 20 and sometimes as high as 22 units per mg. The production of the crystals from such material would accordingly not entail the separation of a highly active principle from a crude mixture, but rather the creation of conditions allowing the crystallisation of a substance already almost pure. It would be analogous rather to the crystallisation of serum-albumin, for example, than to the isolation of such a hormone as adrenaline.

In conclusion we wish to express our indebtedness to Dr H. H. Dale, F.R.S., for his continued interest in this work, and for placing the facilities of his laboratory at the disposal of one of us (D.A.S.) for the animal experiments. We wish especially to thank him for his cooperation in arranging the physiological assay, which could not have been so extensive without his help.

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XLVII. STUDIES IN CARBOHYDRATE METABOLISM.

IV. ACTION OF HYDROXYMETHYLGLYOXAL UPON NORMAL AND HYPOGLYCAEMIC ANIMALS.

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THREE compounds containing three carbon atoms have frequently been considered as possible intermediaries in carbohydrate metabolism: glyceraldehyde, dihydroxyacetone, and methylglyoxal. If the formula of hydroxymethylglyoxal, $CH_2OH.CO.CHO$, is compared with those of these three compounds it is seen that it bears a close relationship to all of them, and may, in fact, be regarded as an oxidation product of any of them. It was therefore considered of some interest to ascertain the action of this compound on the animal organism. This appeared the more desirable since evidence has been advanced by Hynd [1927] and Herring and Hynd [1928] which, it is claimed, supports the view that glucosone, $CH_2OH.(CH.OH)_3.CO.CHO$, is amongst the products formed in the animal body by the oxidation of dextrose under the action of insulin. In particular, they have found that the administration of glucosone to various animals causes the appearance of symptoms similar to those associated with insulin hypoglycaemia in the same species. The suggestion is that glucosone is the compound directly responsible for the appearance of these symptoms. Hydroxymethylglyoxal may be regarded as the simplest compound analogous to glucosone, bearing in fact the same relation to the triose glyceraldehyde that glucosone bears to dextrose. It was therefore of interest to determine whether hydroxymethylglyoxal causes the appearance of toxic symptoms similar to those produced by glucosone.

It may be noted that White and Willaman [1928] have found that hydroxymethylglyoxal is readily fermented by *Fusarium lini*, the only other compounds showing an equal ease of degradation by this mould being dihydroxyacetone and the fermentable hexoses.

Experimental.

The hydroxymethylglyoxal used in the following experiments was prepared by the method of Evans and Waring [1926], with one slight modification. These authors obtained good yields of the compound by oxidising dihydroxyacetone with copper acetate, allowing the mixture to stand for 10 days at ordinary laboratory temperature. We have been unable to obtain a crystalline product under these conditions, but found that the syrup produced rapidly crystallised if the oxidation had been allowed to continue for 30 days at room temperature or for 25 days at 25°. This difference is probably due to the fact that the laboratory temperature was considerably below that which prevails in American laboratories. With this modification we found that the reaction proceeds and the product crystallises just as described by Evans and Waring. It may be mentioned here that these authors found that when solid hydroxymethylglyoxal is dissolved in cold water it exists in a dimeric form, but when heated for 5 minutes at 100° it changes into the monomeric state.

Effect of hydroxymethylglyoxal on normal mice.

Experiments were first of all carried out to ascertain the effect of hydroxymethylglyoxal on normal mice, first with a freshly prepared solution of hydroxymethylglyoxal which had not been heated. The results are summarised in the following table.

Table I. *Effect of injections of unheated hydroxymethylglyoxal solution on mice.*

No. of exp.	Colour of mouse	Vol. of solution injected cc.	Concn. of hydroxy-methyl-glyoxal %	Wt. of hydroxy-methyl-glyoxal g.	Remarks
1	Albino	0·4	15	0·06	Sprawling in 1 minute then lying on side comatose. Breathing shallow, then gasping. Death in 3½ minutes
2	Albino	0·2	15	0·03	Arrest of respirations, convulsions and death in 2 minutes
3	Albino	0·4	7·5	0·03	Convulsions and death in 3 minutes
4	Black and white	0·3	7·5	0·0225	Convulsions, coma and death in 3 minutes
5	Black and white	0·2	7·5	0·015	Drowsiness, tail erected, paddling movements, coma, followed by recovery
6	Black	0·2	7·5	0·015	Erection of tail, convulsions, running movements, coma and death in 3 minutes
7	Black and white	0·2	7·5	0·015	Drowsiness, sprawling, paddling movements. Recovery in 2 hours
8	Albino	0·2	7·5	0·015	Convulsions, coma and death in 4 minutes
9	Black	0·2	7·5	0·015	Convulsions, coma and death in 4 minutes
10	Black	0·2	7·5	0·015	Drowsiness and sprawling, no convulsions. Recovery
11	Black	0·2	7·5	0·015	Drowsiness and sprawling, no convulsions. Recovery
12	Black and white	0·4	0·75	0·003	No symptoms

It will be seen that the lethal dose for a mouse is approximately 0·015 g. In those experiments in which death did not occur rapidly the animals exhibited a train of symptoms resembling those resulting from insulin hypoglycaemia. Thus in mouse No. 5, after a preliminary period during which the respirations were accelerated, the animal became drowsy for a time, then later the respirations became shallow and irregular, the tail was erected and the animal showed running movements. It then became comatose and lay on its side, respiration being barely perceptible, and in fact it was to all appearances moribund. After 20 minutes however, it showed signs of recovery, the respirations increased and the animal sat up unsteadily, the legs sprawling and the tail showing the peculiar stiffening commonly noted in the mouse during insulin hypoglycaemia. It remained in a languid condition for 3 to 4 hours and ultimately recovered completely.

The second series of experiments was carried out with a solution of hydroxymethylglyoxal which had previously been heated to 100° for 10 minutes. The results are summarised in Table II.

Table II. *Effect on mice of injections of hydroxymethylglyoxal solution, heated to 100° for 10 minutes.*

No. of exp.	Colour of mouse	Vol. of solution injected cc.	Concn. of hydroxy-methyl-glyoxal %	Wt. of hydroxy-methyl-glyoxal g.	Remarks
1	Brown	0·2	7·5	0·015	No symptoms
2	Albino	0·4	7·5	0·03	No symptoms
3	Black	0·2	15	0·03	Temporary excitement. No other symptoms
4	Albino	0·2	15	0·03	Transient muscular weakness; sprawling. No other symptoms
5	Albino	0·2	15	0·03	Became inactive for a short time. No other symptoms
6	Albino	0·4	15	0·06	After 8 minutes weakness of extremities. Moved about with difficulty. No convulsions. No coma. Breathing gradually became shallower. Death in 3 hours

It will be seen that the lethal dose of monomeric hydroxymethylglyoxal is 0·06 g., which is about four times the lethal dose of the unheated material. It appears, therefore, that the depolymerisation considerably reduces the toxicity of hydroxymethylglyoxal. Further, the depolymerised substance when administered in sublethal doses does not appear to cause the appearance of symptoms similar to those of insulin hypoglycaemia.

In the preparation of hydroxymethylglyoxal, it appears very difficult to eliminate the last traces of hydrogen sulphide and colloidal sulphur. The sulphur partially separates when the solution is heated to 100°. It seems, however, that hydrogen sulphide plays no part in the production of the toxic symptoms, since no symptoms were produced in control experiments

in which much larger quantities of hydrogen sulphide were employed than were contained in the unheated solution of hydroxymethylglyoxal. Further, the very small precipitate formed by heating the solution had no toxic effects.

Effect of hydroxymethylglyoxal on hypoglycaemic mice.

Ten mice were brought into a hypoglycaemic condition as the result of the administration of 0·2 cc. of standard insulin solution (20 units per cc.), diluted 1 in 25. To five of these mice sublethal doses of an unheated solution of hydroxymethylglyoxal were administered, and to the other five sublethal doses of a solution of hydroxymethylglyoxal previously heated to 100° for 15 minutes. In no case did recovery take place, but the animals recovered promptly after the administration of glucose. Hydroxymethylglyoxal is evidently without effect on insulin hypoglycaemia. It may be added that dextrose is without effect on the toxic symptoms produced by hydroxymethylglyoxal.

Effect of hydroxymethylglyoxal on rabbits.

The above results have all been confirmed by experiments on rabbits. These animals do not recover from hypoglycaemia as the result of the administration of hydroxymethylglyoxal, but rather are made worse, and death frequently follows in 10 or 15 minutes when the unheated material is used. This fatal result was observed even with doses which, when administered subcutaneously to normal animals, caused no obvious symptoms. In this respect the result is analogous to that previously observed with methylglyoxal [Kermack, Lambie and Slater, 1927]. As much as 9 cc. of a 25 % solution of hydroxymethylglyoxal, administered subcutaneously, produced no effect even when the solution had not been heated. When unheated hydroxymethylglyoxal was administered intravenously, 0·25 g. was sufficient to cause asphyxial convulsions and cessation of respiration following a momentary stimulation of the breathing. After generalised convulsions the animal lay on its side, showing running movements and intermittent gasping respirations. Artificial respiration was performed and the animal gradually recovered. On the other hand intravenous administration of 1·25 g. of a solution of hydroxymethylglyoxal, which had previously been heated to 100° for 10 minutes, produced no toxic symptoms. In relation to the results of Hynd, which have been mentioned above, an experiment may be reported here in which enormous doses of insulin (up to 200 units), were administered to rabbits and at the same time very large quantities of dextrose, to prevent reduction in blood-sugar. As expected, no hypoglycaemic symptoms occurred. If insulin produced hypoglycaemic convulsions as the result of the conversion of dextrose into glucosone it appears probable that such symptoms would have been observed in these experiments.

Discussion.

The above experiments demonstrate the highly toxic nature of hydroxymethylglyoxal in its dimeric form. Herring and Hynd [1928] state that 2·4 mg. of glucosone per g. body weight is sublethal for a mouse. In the case of dimeric hydroxymethylglyoxal subcutaneous administration of 0·75 mg. per g. body weight is sufficient to cause death. The symptoms produced by dimeric hydroxymethylglyoxal are similar to those described by Hynd and Herring as being produced by glucosone. On the other hand, 3 mg. of the monomeric form of hydroxymethylglyoxal per g. body weight were necessary to cause death in the mouse and the animal did not exhibit the characteristic train of symptoms above noted. It is surprising to find that the monomeric form, which gives the Schiff's reaction more readily than the dimeric form, and of which the aldehyde groups are therefore in a more active condition, is the less toxic. As pointed out by Olmsted and Logan [1923], the symptoms of insulin hypoglycaemia resemble those produced by asphyxia in the rabbit and cat. It would appear that many substances which interfere with the normal oxidative processes in cells (*e.g.* such as cyanide poisoning, cf. Hess [1921], carbon monoxide poisoning, etc.), tend in the lower animals to produce a train of symptoms which have many features in common, especially within the same species. Among these symptoms are muscular weakness (as shown by sprawling of the limbs or drooping of the head), head retraction, weakness, drowsiness, convulsive seizures with paddling and running movements, and ultimately coma. In the absence of other evidence, it would appear particularly hazardous to conclude that glucosone plays a part in the production of the symptoms of insulin hypoglycaemia because of the resemblance of these symptoms to those produced by insulin. The above experiments show that almost identical effects are produced even more readily by hydroxymethylglyoxal. It is also difficult to explain the non-appearance of such symptoms after the administration of enormous doses of insulin, provided that sufficient dextrose is given to prevent a fall in blood-sugar below the normal. Reference may also be made to the fact that in cases of diabetic coma large quantities of insulin are often given without harmful effect as long as the blood-sugar is not allowed to fall unduly. Even Herring and Hynd [1928] find it necessary to suggest that the sensitiveness of the animal to glucosone depends on the blood-sugar being abnormally low; and so in the absence of independent evidence as to the existence of glucosone in the animal body it does not seem to simplify matters to assume the development of a specific poison to explain the symptoms. It must also be remembered that the symptoms of glucosone or hydroxymethylglyoxal poisoning are not relieved by the administration of dextrose.

In its inability to relieve the symptoms of insulin hypoglycaemia, hydroxymethylglyoxal resembles the majority of compounds containing three carbon atoms of which, so far, only one, namely dihydroxyacetone, has been found to possess the power of bringing about rapid recovery.

SUMMARY.

1. Hydroxymethylglyoxal in its dimeric form is highly toxic to mice and rabbits and in sublethal doses produces symptoms similar to those of insulin hypoglycaemia.

2. In its monomeric form the toxicity is reduced by 75 % and the same train of symptoms is not produced.

3. Hydroxymethylglyoxal, either in the dimeric or monomeric form, is unable to cause recovery from insulin hypoglycaemia.

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XLVIII. STUDIES IN CARBOHYDRATE METABOLISM.

V. EFFECT OF ADMINISTRATION OF DEXTROSE AND OF DIHYDROXYACETONE UPON THE GLYCOGEN CONTENT OF MUSCLE IN DEPANCREATISED CATS.

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Introduction.

It has been shown by Burn and Dale [1924] and by Lambie [1926] that administration of insulin to decerebrated and eviscerated cats results in a more rapid disappearance of dextrose from the blood stream than when no insulin is given. It has further been shown by Best, Hoet and Marks [1926] and by Best, Dale, Hoet and Marks [1926] that a large proportion of the sugar which thus disappears is laid down as glycogen in the muscles.

Much discussion has centred around the question as to whether the essential action of insulin is to convert dextrose into some intermediary substance such as dihydroxyacetone. It therefore seemed desirable to carry out experiments with a view to ascertaining whether dihydroxyacetone causes the deposition of glycogen more readily than does dextrose. Mostowski [1911] showed that the administration of dihydroxyacetone to chickens results in the deposition of glycogen in the liver, whilst in more recent years Isaac and Adler [1924] have claimed that dihydroxyacetone, when injected intraperitoneally into mice and rats, effects more rapid deposition of glycogen in the liver than does dextrose. Cori and Cori [1928] have studied the distribution of glycogen in the livers and muscles of rats fed on these sugars, and found that at the end of 4 hours the rats fed on dextrose deposited 18 % of the sugar absorbed as glycogen in the liver and 25 % as muscle-glycogen, whereas in the case of dihydroxyacetone 21 % was laid down as liver-glycogen and only 15 % as muscle-glycogen. When insulin was administered the distribution of glycogen as between liver and muscle was 6 % and 36 % for dextrose and 2 % and 33 % for dihydroxyacetone, respectively. In both cases dihydroxyacetone failed

to lay down glycogen in the muscles as readily as dextrose but in the case of the liver the percentages were approximately the same. As Cori and Cori point out, these figures cannot be taken as representing the ease of formation of glycogen, since the fraction deposited in the muscles would, other things being equal, be decreased either by increased oxidation or by increased deposition in the liver. No conclusion can therefore be drawn from these experiments as to the relative ease with which the two sugars form muscle-glycogen.

Campbell and Markowitz [1927, 1, 2] have brought forward evidence which seems to prove that in the hepatectomised animal the administration of dihydroxyacetone is unable to cause recovery from the symptoms of hypoglycaemia and that the triose is quite unattacked by the organism under these conditions. These results suggest that dihydroxyacetone is converted in the liver either into dextrose or into some active form of the triose. It is known that dihydroxyacetone can, under certain conditions, be converted by the liver into dextrose, but, whilst this would account for certain resemblances in the physiological behaviour of the two sugars, the differences which they exhibit could not be satisfactorily explained on this hypothesis alone. It therefore seemed possible that experiments on animals in which the liver was intact might afford evidence that an active substance was formed from dihydroxyacetone. The following experiments were therefore carried out upon decerebrated and depancreatised cats in which the liver remained intact.

Experimental.

Cats were employed in all experiments. After brief etherisation the brain was destroyed with a probe through a trephine opening in the skull, and artificial respiration was carried out by means of a cannula inserted into the trachea. Cannulae were also placed in the left jugular vein and the left carotid artery. After withdrawing a sample of blood for analysis (Blood 1 in table), the abdomen was opened and the pancreas was removed. In one experiment only (No. 19 in table) was the animal eviscerated and the liver and kidneys tied off, thus excluding them from the general circulation. After pancreatectomy or evisceration, as the case may be, a second sample of blood (Blood 2 in table) was taken and then transfusion of the sugar under examination was begun. The sugar (dextrose or dihydroxyacetone) was dissolved in saline in the requisite concentration and, by means of the apparatus previously described [Lambie, 1926], was run in at a constant rate through the cannula in the jugular vein. Immediately after the perfusion had begun a ligature was placed round the right femoral artery and the muscles of the right lower limb were removed, cut into small pieces with scissors, weighed *en masse*, and then dropped into boiling 60 % potassium hydroxide solution. After the transfusion had continued for a given time, a third sample of blood (Blood 3 in table) was withdrawn and the left hind limb was removed and added to potassium hydroxide solution after cutting and weighing. The

glycogen content of the muscles was then determined by means of the following method.

Estimation of glycogen in muscle.

The weighed muscle (about 100 g.) was dropped into a conical flask containing hot aqueous 60 % potassium hydroxide (100 cc.) as described above and the mixture heated in a boiling water-bath for about 6 hours when the solid material had been practically completely decomposed and a reddish brown solution remained. On cooling, this solution was poured into a standard 250 cc. flask and diluted with water to the mark. After thorough mixing 5 cc. of the solution were pipetted into a 25 cc. centrifuge tube. Absolute alcohol (20 cc.) was then added and the mixture allowed to stand for 20 hours when the glycogen was completely precipitated. The precipitated glycogen was centrifuged off and washed twice with 66 % alcohol, then with absolute alcohol and finally with ether. It was then dissolved in 2 % hydrochloric acid (10 cc.) and heated on the boiling water-bath for 4 hours. The acid solution was washed into a 25 cc. standard flask and diluted to the mark. The glucose present in a convenient volume of the solution was then estimated by the Hagedorn-Jensen method [1923] and the percentage of muscle-glycogen calculated from this result.

In certain experiments curare was employed in order to paralyse the motor nerve endings of the muscle and eliminate any muscular twitching which might alter the glycogen content of the muscles. A solution, in saline, of the residue formed by evaporation to dryness of an alcoholic extract of the bark of *Strychnos toxifera*, 1 cc. of which had in control experiments been found to paralyse completely the motor nerve endings of the muscles of the lower limb of the cat, was injected in the requisite dose immediately after decerebration had been performed and artificial respiration begun.

As it was advisable to avoid as far as possible stimulation of the pancreas, ergotamine tartrate (5 mg.) was injected 2 hours before the beginning of the experiment with a view to paralysing the terminations of the splanchnic nerves in the liver and thus limiting the initial hyperglycaemia occasioned by the destruction of the brain and the ether anaesthesia, until the pancreas could be removed. It was found, however, that the ergotamine, by paralysing the sympathetic endings in the heart, often caused excessive slowing of that organ and the resulting depression of the circulation was frequently so great that circulatory failure followed the administration of the anaesthetic. When this threatened, adrenaline, 0·2 cc. of 1 in 1000 solution, was injected intravenously in order to save the animal. The results are summarised in Table I.

The total reducing power of the blood, as given in the table, in terms of glucose, was determined by the method of Hagedorn and Jensen [1923], whilst the method of Campbell [1926] was used for the determination of dihydroxyacetone. It was found by the Hagedorn and Jensen method that 1 g. of dihydroxyacetone is equivalent to 1·48 g. of glucose by this method.

Discussion.

The single experiment in which the liver was excluded from the circulation and the animal perfused with dihydroxyacetone showed a slight fall in muscle-glycogen in spite of the fact that the blood at the end of the experiment contained 0·1 % of dihydroxyacetone. This seems to indicate, in agreement

Table I.

No. of exp.	Wt. of cat g.	Sugar perfused	Concn. of sugar in perfusion fluid %	Rate of perfusion per hour g.	Time of perfusion mins.	Total wt. of sugar perfused g.	Blood-sugar (total reducing power) mg. per 100 cc. 1	2	3	Glycogen in muscle mg per 100 g. Before	After	Difference	Remarks
1	2600	Dextrose	40·6	2·02	90	3·04	276	369	1560	234	298	+ 64	Pancreas removed. Liver intact
2	—	,,	40·0	2·0	130	4·30	222	276	318	253	201	− 52	
3	2300	,,	34·5	1·72	117	3·44	—	—	—	476	170	−306	Curare. Pancreas removed. Liver intact
4	—	,,	30·0	1·5	175	4·50	84	106	588	359	238	−121	Ergotamine. Pancreas removed. Liver intact
5	—	,,	30·0	1·5	100	2·50	282	438	—	307	455	+ 148	
6	—	,,	30·0	1·5	135	3·37	254	326	580	574	562	− 12	Ergotamine. Adrenaline. Pancreas removed. Liver intact
7	—	,,	60·0	6·25	106	11·04	144	198	2220	279	206	− 73	
8	—	,,	30·0	1·5	130	3·25	—	104	456	658	687	+ 29	
9	2300	Dihydroxyacetone	30·0	1·5	120	3·00	258	326	870	402	407	+ 5	
10	3200	,,	50·0	2·5	70	2·91	—	318	1560 (0·42 % Di.)	194	242	+ 48	
11	2250	,,	50·0	2·5	90	3·75	140	158	1140 (0·1 % Di.)	592	421	−171	Pancreas removed. Liver intact
12	2270	,,	35·4	1·77	90	2·66	222	343	1020 (0·173 % Di.)	274	288	+ 14	
13	—	,,	40·0	2·0	80	2·66	258	404	1230	149	206	+ 57	
14	—	,,	40·0	2·0	110	3·66	154	220	1710	381	372	− 9	
15	2100	,,	31·5	1·58	120	3·15	—	—	—	274	223	− 51	Curare. Pancreas removed. Liver intact
16	2400	,,	37·4	1·87	90	2·80	—	—	—	499	577	+ 78	
17	—	,,	30·0	1·5	80	2·0	230	426	1008	559	743	+ 184	Ergotamine. Adrenaline. Pancreas removed. Liver intact
18	—	,,	60·0	6·25	90	9·37	272	282	3180	989	564	−425	
19	3450	,,	53·0	2·69	90	4·03	—	254	1140 (0·1 % Di.)	252	232	− 20	Complete evisceration. Liver tied off

The calculated Blood 3 glucose values in experiments Nos. 10, 11, 12 and 19 are 938, 992, 764 and 992 mg. per 100 cc. respectively.

with the conclusion of Campbell and Markowitz [1927, 1, 2], that glycogen is not produced readily from dihydroxyacetone in the absence of the liver, but further experiments are necessary to settle this point.

It will be noted that in certain experiments ergotamine or curare was administered to the animal. As explained above, this was done with the aim of preventing glycogenolysis in the liver or muscles respectively but there was no evidence that the curarised animals gave more constant results than the untreated ones, whilst the animals which had been treated with ergotamine frequently suffered from circulatory failure and usually died if not given adrenaline. Moreover the ergotamine was frequently ineffective in paralysing the splanchnics completely.

It is evident from the figures in the preceding table that no significant difference is observable under the above conditions in the power of dextrose and of dihydroxyacetone to cause deposition of glycogen in the muscles. In the eight experiments with dextrose the mean decrease in muscle-glycogen from the beginning to the end of the perfusion period was $0{\cdot}040 \pm 0{\cdot}051$ %, whilst when dihydroxyacetone was used the decrease was $0{\cdot}027 \pm 0{\cdot}054$ %. It is obvious, however, that in individual experiments a change in the glycogen is quite considerable and the question arises as to whether this can be correlated with any of the other factors which vary from experiment to experiment.

Inspection shows that with the exception of experiment No. 18, a marked fall in glycogen is usually associated with a lower initial blood-sugar, whilst a marked rise in glycogen is usually observed with a high initial blood-sugar. The figures (including Exp. 18) were therefore examined statistically, taking the 13 experiments in which the initial blood-sugar had been determined, in order to find out whether any correlation existed between the level of initial blood-sugar and the change in the muscle-glycogen. The value of the partial correlation coefficient between initial blood-sugar level and change in muscle-glycogen for a constant initial glycogen content was found to be $-0{\cdot}30 \pm 0{\cdot}15$. This correlation coefficient would have been considerably greater but for experiment No. 18. It may be noted that in this experiment an unusually high initial muscle-glycogen existed and the large fall in glycogen content may be related to this factor. Because of the comparatively small number of animals (13), the probable error of the correlation coefficient is high and too much emphasis must not be laid on the result, but the above experiments suggest that the level of the initial blood-sugar in such a perfusion probably plays a rôle in determining the alteration in glycogen. This may be due to the stimulation of the pancreas, in the short time that elapses before it is excised, so that it secretes insulin and in this way brings about the deposition of glycogen.

Whilst these experiments do not prove conclusively that dihydroxyacetone cannot cause deposition of glycogen in the muscles more readily than dextrose, they show that there is no striking tendency towards such glycogen deposition and to this extent are in accord with the observations of Cori and Cori which have been mentioned above. In some experiments, the amount of dihydroxyacetone administered was certainly large enough to effect a definite rise in muscle-glycogen provided the triose readily polymerised to form the polysaccharide. In experiment No. 18, in which 9 g. of dihydroxyacetone were administered, a fall of glycogen occurred of $0{\cdot}425\,\%$, which is by far the greatest fall observed in all experiments, and further, the four experiments in which a fall took place with dihydroxyacetone are those in which the animal received the largest amounts of the triose, and the animal which received the smallest amount of dihydroxyacetone showed the biggest rise. If the correlation coefficient between the amount of triose administered and the change in glycogen be calculated it is found to be $0{\cdot}95 \pm 0{\cdot}02$, which indicates a strong relation between fall in glycogen content and the amount of triose given. As mentioned above, too much emphasis cannot be laid upon the exact figure, because of the comparatively small number of animals used. When the experiments in which dextrose was administered are examined in a similar way, the correlation coefficient is found to be $0{\cdot}56 \pm 0{\cdot}15$. This also indicates the important effect which a large amount of dextrose has in causing the muscle-glycogen to fall. We do not at present propose to discuss the various theories which may be advanced to explain this remarkable result. Since the amount of carbohydrate injected has this effect upon the

disappearance of glycogen from muscle, it is obvious that no conclusion can be drawn, from experiments of this type, regarding the comparative ease with which glycogen can be laid down in muscle.

SUMMARY.

1. When dihydroxyacetone is administered intravenously to decerebrated cats from which the pancreas has been removed, the change in muscle-glycogen depends largely upon the amount of triose administered. With small quantities an increase tends to occur, with large quantities a decrease.

2. When dextrose is administered under similar conditions, results of an analogous type are obtained, although, in this case, the relation between the amount of glucose administered and the change in glycogen may not be so great.

3. The level of the initial blood-sugar, before pancreatectomy, appears to have some effect upon the change in muscle-glycogen, high initial blood-sugars tending to cause an increase. This is probably due to stimulation of insulin secretion by the pancreas.

4. From the experiments, no evidence could be obtained that muscle-glycogen was more readily formed from dihydroxyacetone than from dextrose in presence of the liver.

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XLIX. THE ANTICOAGULANT ACTION OF ANTITHROMBIN.

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(*Received April 24th, 1929.*)

The presence of a substance in blood, which inhibited coagulation, was noted by early workers on blood coagulation. Schmidt [1892] stated that this substance was present in all cells. To it the term anti-fibrin-ferment was applied by J. Mellanby [1908] and antithrombin by Howell [1910]: the former, who found that the anticoagulant action was abolished by heating to 70°, observed that complete disappearance of thrombin could not be effected by the addition of an excess of antiferment and therefore concluded that the union of antiferment with ferment (thrombin) followed the law of mass action. According to Howell [1912] antithrombin enters into combination with prothrombin and also with thrombokinase, but does not combine with thrombin [cf. Bordet, 1920].

The anticoagulant action of blood upon a mixture of fibrinogen (plasma) and thrombin is observable in the presence of sodium citrate. All the experiments about to be described were made with citrated solutions. Under these conditions of experiment thrombokinase does not convert prothrombin into thrombin: the possibility of the coagulation time being affected by any concomitant interaction of thrombokinase and prothrombin, occurring in the plasma employed, is thus avoided. The action of the anticoagulant must, therefore, be exerted either upon fibrinogen or upon thrombin and is presumably similar to the effect of simple dilution of one or other of the solutions of these two substances employed.

The action of serum in producing delay of coagulation in presence of sodium citrate, is illustrated by the following two experiments (quantities of liquid being given in cc.)[1]:

Table I.

Exp.	Plasma	Serum	0·85 % NaCl	Thrombin	Onset of coagulation
1	0·08	—	0·014	0·026	2·5 minutes
2	0·08	0·014	—	0·026	4·5 ,,

It will be observed that the coagulation time is nearly doubled by the addition of a relatively small amount of serum.

A similar result is obtained if plasma is employed instead of serum.

[1] As source of thrombin the venom of *Echis carinatus* [cf. Barratt, 1913] was used. I am indebted to Sir Charles Martin, F.R.S., Director of the Lister Institute, for kindly providing me with a supply of this venom.

The anticoagulant was, however, found to be present in plasma in amount too small for experimental work: in particular the effect upon fibril formation, which it was desired to observe, could not be ascertained, the changes noted being slight and indefinite.

An attempt was therefore made to determine if the anticoagulant substance could be extracted from plasma. Ultimately it was found that the complete disappearance of this substance, which occurs on heating plasma or serum to 70°, does not take place in presence of a large excess of distilled water. Making use of this circumstance some measure of success was obtained by the following method of extraction which was employed for Exps. 3 to 6, given below.

5 cc. of human plasma were added to 45 cc. of distilled water and the diluted plasma so obtained was poured into 450 cc. of distilled water at 100°. After being kept at about 90° for 5 minutes, the mixture was cooled to 70°, at which temperature it was allowed to evaporate. In the course of $2\frac{1}{2}$ hours its volume was reduced to 20 cc. This was put into a parchment thimble and allowed to dialyse into a litre of distilled water for 48 hours. The liquid contained in the parchment thimble was then evaporated to dryness. The residue was extracted with distilled water, in which it in part slowly dissolved, the insoluble portion being separated by centrifugalisation and the supernatant liquid again evaporated to dryness. The residue thus obtained was then extracted with distilled water, more insoluble material appearing. The process of extraction was repeated several times until at length no further insoluble precipitate was obtainable. In this way a small amount of soluble residue forming a brownish hyaline scale was obtained. For the separation of insoluble protein removal of salts by dialysis was found to be essential.

A portion of this soluble extract, dissolved in 0·85 % NaCl solution, was mixed with citrated plasma. To the mixture thrombin was added (Exp. 3), a control experiment, in which 0·85 % NaCl solution free from extract was used, being also made (Exp. 4). These experiments were repeated with a slightly larger amount of extract (Exps. 5 and 6). The figures in brackets in Table II represent the amount of plasma corresponding to the extract employed.

It will be seen that in each case the addition of extract caused delay in the time of appearance of fibrils.

Table II.

Exp.	Plasma cc.	Extract cc.	0·85 % NaCl cc.	Thrombin cc.	Time of appearance of fibril mins.
3	0·05	—	0·06	0·01	20
4	0·05	·06 (0·25)	—	0·01	56
5	0·05	—	0·06	0·01	21
6	0·05	·06 (0·4)	—	0·01	120

In order to determine whether this delay is due to an action exerted upon fibrinogen or upon thrombin, the effect upon the fibrils of the coagulum was

noted and compared with that occurring (1) when the plasma employed is diluted, and (2) when the thrombin added is similarly diluted: in the former case, in which the concentration of fibrinogen is diminished, the fibrils become short and fine, scanty and barely visible; in the latter, in which the concentration of thrombin is lowered, they are also reduced in number, but exhibit increase in length and thickness [Barratt, 1921].

In Table III the condition of the fibrils in Exps. 3 to 6 is given.

Table III.

Exp.	Approximate number of fibrils in field	Length	Thickness
3	3000	15μ to 40μ	$0{\cdot}1\mu$ to $0{\cdot}2\mu$
4	1000	20μ to 50μ	$0{\cdot}1\mu$ to $0{\cdot}2\mu$
5	4000	30μ to 60μ	$0{\cdot}1\mu$ to $0{\cdot}2\mu$
6	1200	75μ to 170μ	$0{\cdot}2\mu$ to $0{\cdot}4\mu$

It will be seen that a decrease in number of fibrils occurs when extract is added, accompanied by an increase in length and thickness. The anticoagulant substance thus causes diminution in the amount of free thrombin present and is, therefore, an antithrombin in the sense of causing such decrease.

It may be observed that the only means at present available of deciding whether the action of antithrombin upon a mixture of fibrinogen and thrombin in presence of sodium citrate is exerted upon fibrinogen or upon thrombin appears to be by noting the accompanying change of type of fibril formation. In this way an unequivocal indication of the mode of action of the anticoagulant is obtained.

Summary.

(1) Experiments were made to determine the effect of antithrombin, obtained from human blood-plasma after removal of precipitable proteins, upon the formation of fibrils in the coagulation of fibrinogen.

(2) It was found that the anticoagulant action of antithrombin upon a mixture of fibrinogen (citrated plasma) and thrombin *in vitro* is exerted upon thrombin, which becomes diminished in amount: no evidence of any action upon fibrinogen was observed.

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L. ON CARRAGEEN (*CHRONDRUS CRISPUS*).

IV. THE HYDROLYSIS OF CARRAGEEN MUCILAGE.

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(*Received April 25th, 1929.*)

The chemical composition of carrageen has been the subject of investigation from time to time by various authors [*e.g.* Flückiger and Obermayer, 1868; Bente, 1875, 1876]. More detailed investigations were carried out by Haedicke, Bauer and Tollens [1887], who established the presence of fructose and estimated the galactose content at 28 %. Sebor [1900] confirmed the presence of galactose and fructose, found small quantities of pentosan and claimed to have found evidence of glucose, a question which will be referred to again later in the present paper.

All the above authors assumed the mucilage which is obtained by extracting the weed with water to consist of a single polysaccharide. Later, however, it was shown by Haas and Hill [1921] that the extract is in fact a mixture of two substances both of which are carbohydrate esters of sulphuric acid of the following type:

$$\mathrm{R}\left\langle\begin{matrix}\mathrm{OSO_2O} \\ \mathrm{OSO_2O}\end{matrix}\right\rangle\mathrm{Ca}$$

in which R stands for the carbohydrate radicle. These two ethereal sulphates, which were described as hot and cold extracts (H.E. and C.E. respectively), differed primarily in their solubilities and in the fact that the H.E. in 3 % solution forms a gel whereas the C.E. forms instead a viscous liquid. The present investigation is the outcome of an attempt to isolate the carbohydrate complex free from sulphate, but, as the result of a number of trials with various strengths of acid, it was found that the conditions which favour the separation from either H.E. or C.E. of the sulphate residue involve the complete breakdown of the carbohydrate complex.

It has been known that carrageen mucilage cannot be heated with dilute acids such as lemon juice or vinegar without sacrificing its gelatinising power. This observation seemed of some interest, and experiments were accordingly undertaken with the object of determining the strength of acid which would just destroy the gelatinising power of H.E. It was found that when a 2 % solution of H.E. was heated at 80° for 45 minutes with 0·15 *N* sulphuric acid the resulting liquid had acquired reducing properties, but, contrary to expectation, this was not due to the formation of free sugar but to the production of a non-dialysable product which proved to be still an ethereal sulphate.

In an actual experiment 250 cc. of 2 % H.E. were mixed with 250 cc. of 0·15 N sulphuric acid and heated on a water-bath for 45 minutes at 80°. The contents of the flask were then cooled and neutralised with calcium carbonate and, after filtration, evaporated to small bulk *in vacuo* and dialysed against distilled water for 2 days, then against running tap water for another 2 days, and finally once more against distilled water for a further 24 hours: the contents of the dialyser were then evaporated to dryness. The resulting product was designated the "residue."

The outer water from the first two days' dialysis was collected, evaporated to small bulk, filtered, heated to boiling, treated with a hot solution of baryta and left to stand overnight. After filtering, the excess baryta was precipitated with carbon dioxide, and the liquid heated to boiling, filtered and evaporated to dryness over a water-bath; this product was designated the "dialysate." As the residue was found to be slightly dialysable, the dialysate was further purified by being once again put into the dialyser, in this case only the first 12 hours' dialysate being collected and evaporated to dryness.

Examination of residue and dialysate.

Residue. This, consisting of light yellow horny scales and having an ash content of 19·75 %, was found to reduce Fehling's solution and to be optically active, specific rotation + 38·3°. It gave a positive Selivanoff reaction indicating the presence of fructose; the presence of pentose was also established by Bial's reagent and the production of furfural on heating with 12 % hydrochloric acid. On oxidation with nitric acid, crystals of mucic acid were obtained proving the presence of galactose. The solution gave no precipitate with barium chloride until after hydrolysis with hydrochloric acid, when a precipitate of barium sulphate was obtained which corresponded to a sulphate content of 26·72 %. An estimation of the sulphate contained in the ash obtained by incinerating a sample of the same material gave a value of 13·66 % calculated on the dry weight of the material. From these two figures the presence of an ethereal sulphate may be regarded as established [cf. Haas, 1921].

Dialysate. This was obtained as a brown, gummy material which it was not found possible to purify. It gave no precipitate with barium chloride until after hydrolysis with hydrochloric acid, from which it was inferred that it was also an ethereal sulphate. The substance gave the colour reactions for pentose and for fructose, but on oxidation with nitric acid yielded no mucic acid, showing the absence of galactose. It did not reduce Fehling's solution until after hydrolysis and was optically inactive.

The fact that the reducing properties were confined to the residue, which was unable to pass through a parchment dialyser, while the dialysable product had not reducing properties, showed that no free sugar had been produced as the result of the mild conditions of hydrolysis described above, since such sugars, if formed, would have passed out of the dialyser into the outer water.

From the description of the dialysate and residue given above it will be seen that they differ from each other and from the original H.E. The following table summarises the characters of the three substances:

	Dialysate	Residue	H.E.
Pentose (Bial's reagent)	+	+	+
Galactose (by mucic acid test)	–	+	+
Fructose (by Selivanoff)	+	+	+
Reduction of Fehling's solution	–	+	–
Optical activity	Inactive	Dextrorotatory	?*

* The optical activity of this material could not be satisfactorily determined owing to the fact that a sufficiently strong solution for the purpose is too opaque.

The occurrence of glucose in carrageen mucilage.

The presence of glucose in carrageen was originally suggested by Sebor [1900] on somewhat slender evidence; subsequently Müther and Tollens [1904] claimed to have established the presence of glucose on the basis of an analysis of the silver salt of a presumed saccharic acid. Since the silver content of saccharic acid is the same as that of mucic acid, a determination of the silver content furnishes no proof of the presence of glucose. It will be seen from the table given above that both dialysate and residue give the Selivanoff reaction for fructose before hydrolysis. When hydrolysed by heating on a water-bath for 3 hours with 5 % hydrochloric acid, however, the resulting solutions no longer gave the Selivanoff reaction, but on treatment with phenylhydrazine the residue alone gave a precipitate of glucosazone. Since the negative Selivanoff reaction showed the absence of fructose, the osazone could only have been formed from glucose.

In order to obtain further evidence in support of the presence of glucose, an attempt was made to prepare the acid potassium saccharate by oxidation with nitric acid; here a difficulty was encountered from the fact that, owing to the high calcium content of the material, the products of oxidation, namely mucic, oxalic and possibly saccharic acids, were obtained in the form of their sparingly soluble calcium salts. This circumstance makes it probable that saccharic acid, if present in small quantity, would escape detection, and in fact preliminary experiments failed to reveal any of this acid. To overcome this, recourse was had to the oxidation of a sample of the potassium salt of the H.E. For this purpose the mucilage was boiled on a water-bath with potassium carbonate, and subsequently filtered from the calcium carbonate formed. 2 g. of the resulting potassium salt were then oxidised with nitric acid, filtered from the crystals of mucic acid produced, and treated with anhydrous potassium carbonate and acetic acid in the usual manner. After the solution had stood for some days, perfectly typical crystals of acid potassium saccharate were obtained. This experiment, taken in conjunction with that in which glucosazone was obtained from the residue after the destruction of the fructose, establishes conclusively the presence of glucose in the mucilage of carrageen.

Alkaline hydrolysis.

A further attempt was made to isolate the carbohydrate residue by alkaline hydrolysis of C.E.[1] For this purpose various concentrations of aqueous sodium, potassium and barium hydroxides were successively tried both at the temperature of the water-bath and at higher temperatures in an autoclave; but, as in the case of acid hydrolysis, it was found that any attempt to split off the sulphate complex was accompanied by a breakdown of the carbohydrate with the formation in this instance of lactic acid and other products. Moreover, an examination of the products of alkaline hydrolysis revealed the fact that the quantitative liberation of the sulphate is a very slow process, as is shown by the following experiment. Portions of C.E. weighing 0·7 g. were heated with 40 cc. 3 % sodium hydroxide (free from sulphate) in an autoclave at 110° for varying lengths of time and the amount of free sulphate produced was estimated. The results obtained are presented below in tabular form. From the figures a curve was constructed which by extrapolation indicated that complete hydrolysis should have been effected in 54½ hours. Actual experiment showed, however, that out of a possible 30 % of sulphate only 20·5 % could be so obtained. This suggests that a portion of the sulphate is united more firmly than the rest, since it resists hydrolysis by 3 % sodium hydroxide at 110°.

Time heated hours	Sulphate obtained %	Time heated hours	Sulphate obtained %
1	10·42	9¼	18·28
2	12·16	11½	18·18
3	14·78	16¼	19·86
7	17·00		20·00

This conclusion is in agreement with the observations made upon acid hydrolysis where it was found that, while appreciable quantities of sulphate could be set free by a few minutes' boiling with dilute mineral acid, complete hydrolysis could only be effected by 6 hours' boiling with 4 *N* hydrochloric acid.

Action of alcoholic potassium hydroxide on H.E.

12 g. H.E. were dissolved in 160 cc. water and 320 cc. 3 % alcoholic potassium hydroxide added. The H.E. was precipitated, but in spite of this the whole was heated on a water-bath under a reflux condenser for 15 hours. Examination of the residue showed that three different substances had been produced, a portion soluble in cold water, another soluble in hot water only and a third which was insoluble in either hot or cold water. That none of these three was the sulphate-free carbohydrate complex was shown by the fact that they all contained a higher percentage of ash than the original material. The hot water-soluble portion was found to have a gelatinising

[1] In this case the C.E. was selected for experiment since it is an ethereal sulphate of the same type as H.E. but takes considerably less time to prepare.

power five times as great as that of the original substance. It was shown to be an ethereal sulphate and is probably the potassium salt of the original H.E.; previous attempts to prepare this salt by double decomposition from the original mucilage, which is a calcium salt, had resulted in a compound which also had a greater gelatinising power than the original but contained, like the original, a certain percentage of nitrogen, whereas the product here obtained was nitrogen-free. It was not further investigated.

Summary.

(1) The loss of gelatinising power resulting from mild hydrolysis of carrageen is not accompanied by production of free sugars although reducing properties are developed.

(2) The solution resulting from mild hydrolysis of carrageen contains two ethereal sulphates, which can be separated by dialysis. The product which is retained by the dialyser has reducing properties; the substance which passes through has no reducing power.

(3) The presence of glucose has been established in carrageen mucilage.

(4) Alkali hydrolysis of a cold water extract is shown to result in complete breakdown of the carbohydrate complex, and it was found that a quantitative separation of the sulphate could not be effected by this means.

The authors acknowledge their indebtedness to Miss K. J. Bardsley, M.Sc., for a preliminary study of the behaviour of carrageen under varying conditions of acid hydrolysis.

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LI. THE ISOLATION OF A CARBOHYDRATE DERIVATIVE FROM SERUM-PROTEINS.

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(Received April 29th, 1929.)

Many investigators in the past have, at different times, called attention to the fact that preparations of proteins made by them persistently gave positive Molisch or orcinol tests. In no case, however, was any satisfactory evidence brought forward in support of the view that carbohydrate substances could be separated from proteins until Seemann [1898] obtained glucosamine from egg-albumin as the hydrochloride, after acid hydrolysis of the protein. Numerous other investigators have reported the isolation of osazones as a result of treating the acid hydrolysates of various proteins with phenylhydrazine hydrochloride in the customary manner. Thus Mörner [1893] obtained from serum-globulin an osazone melting at 170°-172°. Pavy [1895], Krawkoff [1896] and Jacewicsch [1897] also reported positive findings, whilst Langstein [1902] succeeded in isolating benzoylglucosamine after having benzoylated the mixture obtained by hydrolysing crystalline serum-albumin.

A very large amount of work has also been done upon the mucins, clearly proving that they are glucoproteins and establishing their conjugate character.

In the case of the simpler proteins—especially the albumins and globulins—agreement is by no means so general. Whilst researches such as those quoted above seem to indicate the probable presence of carbohydrate groups as integral constituents, the criticism is always possible that the starting material was not put through a sufficiently rigorous process of purification. Moreover, evidence of a more indirect nature has been sought by examining the power of hydrolysed proteins to reduce alkaline copper solutions and whilst many investigators have claimed positive findings in this direction the results of others have been directly opposed [Hofmann and Pregl, 1907]. Abderhalden, Bergell and Dörpinghaus [1904] deny the presence in serum-albumin of any carbohydrate capable of giving a positive Molisch reaction. Among such contrary findings it is difficult to draw any conclusion. The present author would suggest that the lack of unanimity is very probably due in large measure to the differing experimental conditions. Most previous workers have employed relatively concentrated acid to effect hydrolysis of the proteins. Under these conditions secondary reactions are liable to occur, involving amino-acids

and any carbohydrate that may be formed, and resulting in humin production. It is significant that in nearly all cases the carbohydrate recognised, or claimed to have been present, is glucosamine or an amino-sugar. Now glucosamine gives neither the Molisch nor the orcinol tests and therefore if these tests were originally positive the protein must also have contained some other carbohydrate substance.

Experimental.

In the present investigation very carefully purified proteins have been used and a method of hydrolysis employed which, whilst effective in breaking up the protein, had little or no action upon the carbohydrate substances formed.

Preparation of the serum-proteins.

Freshly drawn horse's blood was allowed to stand at 0° for 24 hours. The expressed serum (2 vols.) was separated off and centrifuged. It was then diluted with 5 vols. of water and 3 vols. of saturated ammonium sulphate were added. The small precipitate was filtered off and discarded. Ammonium sulphate was added to the remainder until half saturation. The precipitated globulin was separated by suction and the albumin precipitated by fully saturating with solid ammonium sulphate.

These two precipitates were purified by eight successive reprecipitations from large volumes of solution, the end fractions being discarded.

Each was then denaturated by addition of 5 vols. of alcohol and 30 cc./litre of a salt buffer mixture[1] (p_H 4·7) to its aqueous solution. After removal of the alcohol, the denaturated protein was washed by repeated extraction with large volumes of distilled water (5 litres to 150 g. protein) filtering by suction between each washing. Dehydration was finally accomplished by alcohol and absolute ether. The preparations so obtained were pure white in colour. Coloured filter papers were employed throughout so that any fine particles adhering to the precipitates could be easily seen and removed. Preparations of both albumin and globulin used in reasonable quantity were found to give a faint, but distinctly positive, Molisch reaction. The colour appeared to form somewhat slowly and was most intense after about half an hour's standing. Possibly hydrolysis in the presence of the acid was occurring, liberating the reactive agent.

Isolation of a carbohydrate derivative.

Hydrolysis was accomplished by boiling under a reflux condenser 150 g. of albumin or globulin with an equal weight of crystallised baryta and ten times its weight of water.

[1] Made up as follows.

Sol. A. 40 g. potassium sulphate, 202·5 g. sodium sulphate (cryst.), 360 cc. water heated to effect solution, then cooled. Sol. B. Normal acetic acid. Sol. C. Normal sodium acetate. Equa volumes of A, B and C mixed: $p_H = 4{\cdot}7\text{–}4{\cdot}8$.

After $3\frac{1}{2}$ hours the golden-coloured solution, which gave a strongly positive Molisch reaction, was cooled, filtered from a little insoluble material and the bulk of the barium removed by passing a stream of carbon dioxide through the solution at 60°.

After filtration, saturated lead acetate was added, and the slight precipitate filtered off and discarded, as it was invariably found to give no Molisch reaction. The carbohydrate was precipitated, however, by addition of ammonia which was continued as long as any precipitate formed. The filtrate gave no Molisch reaction, showing that separation was complete.

The lead-ammonia precipitate was filtered by suction, washed repeatedly on the filter with weak ammonia, finally with distilled water, and then ground into a cream with water. Upon acidifying with acetic acid most of the precipitate dissolved; the insoluble matter, which was usually pigmented, could be filtered off and discarded as it gave no Molisch reaction. Addition of excess of ammonia to this filtrate once more precipitated the carbohydrate. It was found by experience that much difficulty could be saved at later stages by repeating this washing and reprecipitation of the lead compound several times.

Finally it was suspended in water and decomposed by a stream of carbon dioxide at 60°. The point at which decomposition is complete can be easily ascertained with practice, for at this stage the suspended lead carbonate rapidly sinks to the bottom of the containing vessel.

The liquid was filtered. It was light yellow in colour, gave an intense Molisch reaction and pink biuret reaction, showing that it still contained protein substances. Most of the latter were removed by addition of mercuric chloride solution (saturated). It was necessary to add the reagent until further addition caused no turbidity and then to allow the mixture to stand in the laboratory for several days—10 or 12 preferably—as precipitation continued slowly for some time. The filtered liquid was now treated with hydrogen sulphide to remove excess of mercury and lead and after filtration a stream of air was drawn through to remove excess of gas. At this stage the solution was colourless or very pale yellow. It still gave a faint biuret reaction (pink) and an intense Molisch reaction but did not give any trace of turbidity with the following protein precipitants: uranium acetate, sodium tungstate, copper acetate. Phosphotungstic acid in 5 % sulphuric acid gave a white precipitate. This contained the carbohydrate but precipitation was not complete until a large excess of the reagent was present. The final stages of isolation took place as follows. The clear filtrate from the mercuric sulphide was concentrated under reduced pressure to a small volume. Four volumes of methyl alcohol were then added, followed by 5 vols. of absolute ether.

The carbohydrate material was precipitated as a sticky mass which adhered to the sides of the vessel and stirring rod. By leaving it in contact with absolute ether, its character changed completely, for on dehydration it broke up into a fine white powder, having an almost crystalline appearance.

After 5 or 6 reprecipitations by means of methyl alcohol and ether, it was

found that the nitrogen content remained constant at 4·4 to 4·9 % (in place of 5·5 % after the first precipitation). The material at this stage still gave a pink biuret reaction suggesting admixture with protein substances.

Much biuret-giving material was eliminated by addition of glacial acetic acid to the concentrated aqueous solutions and, after 2 or 3 precipitations by ether, the anhydrous material was found to have a constant nitrogen content of 4·1 %. The products isolated from albumin and globulin respectively were apparently identical. The yield from each protein was just under 2 % of pure material.

Microanalysis[1] yielded figures which, after correction for a slight amount of ash (about 0·2 %), agreed with the formula $C_{12}H_{23}O_{10}N$. A small but variable amount of chlorine was also present (about 3 %) and this has been allowed for in reckoning the percentages.

Carbohydrate from albumin.

Prep. I. Total N 3·99, 4·28, 3·94 %.
Prep. II. C 41·57, 42·89; H 7·26, 7·61; N 3·99, 4·14 %.
Calcd. for $C_{12}H_{23}O_{10}N$: C 42·21, H 6·79, N 4·10 %.

Carbohydrate from globulin.

Prep. I. Total N 3·98, 4·17 %.
Prep. II. C 41·73, 42·71; H 7·01, 6·75; N 4·19 %.
Calcd. for $C_{12}H_{23}O_{10}N$: C 42·21, H 6·79, N 4·10 %.

Properties of the carbohydrate derivative.

The substance isolated appears to be a nitrogen-containing polysaccharide. It is extremely hygroscopic, dissolves readily in water, giving a pale yellow solution, but is without measurable optical activity. The Molisch reaction is intense, the biuret reaction negative and the orcinol test gives merely a greenish-red colour quite different from the reaction of glycuronic acid or the pentoses. Solutions of the substance do not reduce Fehling's solution but do so after hydrolysis with acid.

On account of these properties it was thought desirable to obtain an accurate determination of the molecular weight. The cryoscopic method was used, the solvent being water, and the following results obtained:

Carbohydrate from globulin.

$c = 6{\cdot}406$; $\Delta = 0{\cdot}348°, 0{\cdot}347°, 0{\cdot}350°$; Mean 0·348. $M = 340{\cdot}6$.

A disaccharide $C_{12}H_{23}O_{10}N$ would have $M = 341$. The substance is correctly represented, therefore, by its empirical formula.

Identification of the component sugars. An attempt was made to isolate the constituent sugars in the following way. 0·595 g. substance (from serum-globulin) was heated with 18·3 cc. of 20 % hydrochloric acid for 6 hours in

[1] All microanalyses were performed either by Dr Ing. A. Schoeller of Berlin or by Mr A. R. Colwell of the Biochemical Laboratory, Cambridge.

a sealed tube at 100°. The solution contained some dark tarry matter which was filtered off and the filtrate concentrated *in vacuo* over sulphuric acid and sodium hydroxide until nearly dry. Some crystalline material which separated was removed, washed with a little alcohol and dried for analysis. It proved to be a hydrochloride containing nitrogen.

Analysis (micro):	C	H	N	Cl
Found	33·02	6·41	6·25	16·31
Calcd. for $C_6H_{14}O_5NCl$ ... ... (glucosamine hydrochloride)	33·40	6·54	6·50	16·45

A solution of this substance when heated on the water-bath for 2½ hours with phenylhydrazine hydrochloride and sodium acetate yielded an osazone which was identified as glucosazone microscopically and by its melting point 202°–206°. It was concluded therefore that the substance was *glucosamine hydrochloride.* The yield was 0·122 g. or 41 % of the theoretical.

The residual solution from which the glucosamine had separated was evaporated to dryness and the residue repeatedly extracted with alcohol, the alcoholic extracts being combined, and the alcohol evaporated on the water-bath. The residue was taken up in a little water and to this solution phenylhydrazine hydrochloride and sodium acetate were added and the mixture left in a boiling water-bath. An osazone formed resembling glucosazone in microscopical appearance. The crystals were washed with 20 % alcohol then dissolved in hot absolute alcohol; water was added until turbidity resulted and the solution allowed to cool. Typical glucosazone crystals formed, having melting point 204°-205°. It was thus shown that the sugar associated with glucosamine in this carbohydrate derivative obtained from serum-globulin was a hexose capable of yielding glucosazone and therefore either glucose, fructose or mannose.

Reducing power and optical activity of hydrolysate.

In order to gain further evidence concerning the nature of the hexose some periments were experformed in which the carbohydrate material was hydrolysed by boiling with acid and the optical rotation and reducing power of the solution determined when hydrolysis was complete.

The method employed for determining reducing power was that of Willstätter and Schudel [1918] where the active agent is sodium hypoiodite which distinguishes between aldoses and ketoses, only the former being oxidised. 0·4689 g. of the carbohydrate from globulin was dissolved in 50 cc. of N HCl and the mixture boiled. At intervals, samples were taken and reducing power determined by the iodine method.

Time (hours)	Reducing power expressed as glucose (mg.)
0	—
½	78·7
1	229·0
2	425·4
3½	459·7
5	481·2
Theoretical possible	493·5

Within 5 hours hydrolysis was practically complete, the reducing power being 97·5 % of theory (all expressed as glucose). The remaining solution, amounting to 14 cc. in all, was neutralised by sodium hydroxide, filtered and its rotation observed.

Rotation in 2 dm. tube	$= + 0{\cdot}40°$
Concentration (from reducing power)	$= 567{\cdot}3$ mg./100 cc.
$[\alpha]^{18°}_{5461}$	$= + 35{\cdot}28°$.

Assuming glucosamine and mannose to be present in equal proportions, calculation from the known constants gives, for the mixture, an estimated[1] $[\alpha]_{5461}$ of $+ 38{\cdot}64°$, whilst for glucosamine + glucose $[\alpha]_{5461} = + 61{\cdot}27°$. Considering the difficulties of measurement, using such small quantities, the agreement is sufficiently close. It may be pointed out that since both components of the disaccharide are oxidised in the iodine method the possibility of fructose being present is excluded. The substance must therefore consist of glucosamine together with either glucose or mannose and the above polarimetric evidence strongly suggests that the hexose is mannose.

Isolation of mannosephenylhydrazone.

For the isolation of mannosephenylhydrazone from the hydrolysis mixture 1 g. carbohydrate material was boiled with 10 cc. *N* HCl for 5 hours. When cold, the brownish-yellow liquid was neutralised to litmus paper by the very cautious addition of sodium hydroxide solution. It was then filtered and warmed to about 37°, and solid phenylhydrazine hydrochloride (0·4 g.) and crystalline sodium acetate (0·38 g.) were added. Solution took place very rapidly, whereupon the liquid was again filtered, allowed to cool and gently stirred as soon as crystals began to separate. The tube was stoppered and left in ice overnight. The crystalline material (which consisted of extremely fine microscopic prisms or needles) was separated by centrifugation and washed repeatedly, in the same manner, first with distilled water, then with cold 60 % alcohol and finally desiccated over sulphuric acid *in vacuo*.

By elementary analysis and melting point it was identified as mannosephenylhydrazone. Preparations contained a little ash (about 0·9 %), which has been allowed for in calculating the percentages.

Analysis (micro):		C	H	N
Carbohydrate from globulin. Found	...	52·95	6·78	10·85
Carbohydrate from albumin. Found	...	53·11	6·54	10·82
Calcd. for mannosephenylhydrazone ($C_{12}H_{18}O_5N_2$)	...	53·33	6·67	10·37

The substances had M.P. 198–199° and 199–200° (uncorr.) respectively. The values given in the literature for the melting point of mannosephenylhydrazone vary from 195° to 200°. A specimen prepared from pure mannose had M.P. 200°; mixed M.P. with substance isolated 199°.

[1] The factor 1·18 has been used to convert values of $[\alpha]_D$ into those for the line 5461 Å.

Isolation of glucosamine hydrochloride from the carbohydrate obtained from albumin.

The carbohydrate materials isolated from the albumin and globulin respectively agree in having the same elementary composition and molecular weight and also in their physical and general properties. They appear in fact to be identical. Glucosamine was identified in the substance from albumin in the following manner.

0·2 g. of the carbohydrate was boiled for 5 hours with 50 cc. of 10 % hydrochloric acid. The resulting solution was concentrated over sulphuric acid and sodium hydroxide *in vacuo* until crystals began to separate. After standing overnight the crystals were separated from the dark syrupy mother liquor, washed with 97 % alcohol and then dried. They were yellowish white in colour, but after recrystallisation from dilute hydrochloric acid were obtained colourless.

Analysis (micro):	C	H	N	Cl
Found	33·43	6·92	6·49	16·96
Calcd. for glucosamine hydrochloride ($C_6H_{14}O_5NCl$)	33·40	6·54	6·50	16·45

Preparation of the carbohydrate material by tryptic hydrolysis of the protein.

The carbohydrate groups present in the mucins and other glucoproteins have the structure of polysaccharides in which glucosamine and glycuronic acid are united. It has been shown by Levene and La Forge [1913], however, that these substances also contain sulphur, in the form of sulphuric acid in ester linkage with the sugars, whilst the amino-group of the glucosamine is not free, but is acetylated. Since the conditions of hydrolysis obtaining in the work here recorded were such as would probably remove any ester-linked sulphuric acid, were this originally present in the carbohydrate complex obtained from the serum-proteins, and also such as to eliminate any acetyl groups from their union with amino-nitrogen, it was felt that possible criticism should be met by endeavouring to obtain the carbohydrate by other methods.

As identical substances were obtained from the albumin and globulin respectively of horse-serum, in this experiment their separation was omitted. In all other details the preparation of the proteins resembled that previously described, but addition of ammonium sulphate was carried to full saturation and the albumin and globulin filtered off together.

200 g. of the carefully purified and dried mixed serum-proteins were suspended in 2 litres of water together with a little toluene. After standing overnight the protein was soft and evenly dispersed throughout the liquid. Sufficient sodium hydroxide to bring to p_H 8·3 was then added, followed by 10 cc. of Allen and Hanbury's "Trypsin." This enzyme preparation was selected as being the most suitable since it gave an almost entirely negative Molisch reaction; other commercial preparations contained considerable quantities of carbohydrate material. After 2 days' incubation at 38° a further 10 cc. of enzyme solution was added and the p_H readjusted to 8·3.

Hydrolysis was allowed to proceed at 38° for 4 weeks, the mixture being shaken occasionally. It was then filtered through pleated filter papers. The filtrate was clear yellow in colour and gave an intense Molisch reaction. A scheme of isolation was followed identical with that previously described. Much trouble was encountered in the final stages, however, in ridding the carbohydrate of admixed peptone material, and a repetition of the precipitation with lead acetate and ammonia, followed by all the subsequent stages, was necessary before satisfactory analytical figures were obtained. These showed the substance to be identical with that isolated by the baryta hydrolysis method. The yield was slightly smaller (about 1·5 %).

Sulphur was tested for but was found to be absent from the preparation.

Analysis (micro):	C	H	N
Found	42·68	7·39	4·65
Calcd. for $C_{12}H_{23}O_{10}N$	42·21	6·79	4·10

It is clear as a result of these experiments that the carbohydrate substance present in the serum-proteins is of a type quite different from that occurring in the mucins and has nothing in common with the latter save the presence in each of glucosamine as one of the constituent units.

Structure of the carbohydrate derivative.

The molecular weight of the substance isolated is in accordance with that required by the simple formula $C_{12}H_{23}O_{10}N$. Hence it is clear that the substance is of the disaccharide type and not a complex polymer. The absence of reducing properties would in this circumstance suggest a linkage similar to that present in trehalose, involving the two reducing groups of the component sugars.

On the other hand, a union of the α- or β-glucoside type would result in a molecule exhibiting the same behaviour towards copper solutions if, at the same time, the reducing group of one or other of its components was involved in some way—for example, with the amino-group of the glucosamine—so that its potentialities were in abeyance.

In order to gain further insight into the constitution of the compound attention was directed towards the behaviour of the amino-group. Glucosamine and its methyl ester give off nitrogen quantitatively when acted upon by nitrous acid. When the disaccharide was investigated, using the Van Slyke microapparatus, the following results were obtained, from which it is clear that the amino-group, though slowly attacked, is capable of reacting quantitatively to form nitrogen.

Solution (*a*). 11·768 mg. in 2·5 cc.

Reaction time 3 mins. ... 1·77 cc. gave 0·22 cc. N_2 at 763 mm. and 20° = 1·66 % N_2.

Solution (*b*). 50 mg. in 10 cc.

Reaction time ½ hr.	... 2 cc. gave	0·41 cc. N_2 at	769 mm. and	19°	= 2·37 % N_2
,, 1 ,,	... 2	0·56	765 ,,	20	= 3·23 ,,
,, 2 ,,	... 2	0·65	762 ,,	21	= 3·67 ,,
,, 6 ,,	... 2	0·70	760 ,,	20	= 3·99 ,,
				Theoretical	= 4·10 ,,

Hynd and Macfarlane [1926] have recently examined the behaviour towards nitrous acid of a number of amino-compounds. Glucosamine they found to react only slowly in presence of 20 % acetic acid, taking 18 hours for complete decomposition, although its salts like the hydrochloride, or the free base in presence of mineral acid, yielded all their nitrogen as gas within half an hour at 20°.

They conclude from these observations that glucosamine must contain the modified betaine ring suggested by Irvine and Hynd [1913], whilst in the case of its salts, or of the base itself in presence of mineral acid, this linkage is unstable and gives way to form the free amino-group.

In many ways the properties of the carbohydrate compound are in keeping with the suggestion that the nitrogen atom of the glucosamine is involved in a ring of the betaine type, the other hexose being attached in a manner similar to the methyl group in Irvine and Hynd's α-aminomethylglucoside. Like this substance it exhibits a stability towards acid hydrolysis that is quite irreconcilable with a simple glucosidic linkage, and the slowness with which the reaction takes place between the amino-group and nitrous acid in the presence of a weak organic acid like acetic acid suggests that the substance belongs to the "free-glucosamine" type, containing ring-nitrogen rather than that of the glucosamine salts as typified by the hydrochloride.

It is impossible, however, to overlook a point of some difficulty arising at this juncture, should the suggestion be accepted that the nitrogen atom is part of a betaine ring.

The work of Kenner and Earl [1927] has shown that a grouping, in which a nitrogen atom occurs in the quinquevalent state, bears a positive charge. In most cases this will give rise to a negatively charged ion but in the case of a disaccharide such as that described here it is difficult to see how this condition could be fulfilled. It is, however, noteworthy that in all preparations a very great difficulty was encountered in attempting to eliminate traces of chlorine from the compound during its purification, an attempt which, in fact, proved unsuccessful. There was in each case a small quantity of chlorine, amounting to some 2 to 4 %, which could not be removed by the precipitation procedure adopted. This quantity is smaller than would be required by one atom of combined chlorine and was moreover variable, but its presence accords with the view that there was resident somewhere in the molecule a sphere of electropositive influence.

Experiments were next undertaken to determine whether any of the usual enzymes acting on carbohydrates were capable of hydrolysing the compound. Reducing power was determined by the method of Hagedorn and Jensen, at intervals of time up to 4 days. The enzyme preparations were all freshly prepared and the activity of each was tested upon a suitable substrate. In all cases buffer solutions of the appropriate p_H were added to the test and control solutions.

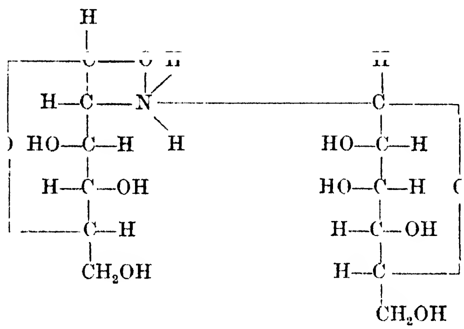
The following results were obtained:

Enzyme	Effect
Emulsin	Nil
Ptylain	Nil
Pancreatic amylase	Nil
Maltase	Slight hydrolysis?

With maltase the observed increase in reducing power was but slightly greater than the experimental error; trehalase, unfortunately, has not yet been available for trial.

The evidence furnished by these enzyme experiments militates against the view that an α- or β-glucosidic linkage is present. It is in keeping with the suggestion that the two sugar molecules are united as in trehalose, or as in the modification of this linkage possible when one constituent is an amino-sugar, in which the betaine ring occurs.

In the absence of further evidence it is perhaps unwise to consider the structure of this carbohydrate as proven. The following formula is, however, put forward with a certain degree of confidence as indicating its probable constitution.



There are two possible ways in which such a structure could enter into combination with the amino-acid complexes of the protein molecule. In the first place attachment might occur through the nitrogen atom, or, secondly, it is possible that condensation might occur between one or other of the hydroxyl groups and some constituent of the protein molecule. In the absence of further knowledge there is no way of deciding between these two alternatives.

Deductions concerning the molecular weights of the serum-proteins.

Both the albumin and globulin of horse-serum yielded about the same quantity of pure carbohydrate material, namely 2 %. Since the carbohydrate possesses a molecular weight of 341, the minimal molecular weight of each protein is given by the expression $M = \frac{341 \times 100}{2} = 17050$. Of course such a calculation can give only approximate figures since it is not possible to determine the percentage content of carbohydrate more exactly than by direct isolation; however, this figure is in quite good agreement with the values recorded in the literature and found by methods very different from that here employed [see Svedberg and Sjögren, 1928; Cohn, Hendry and Prentiss, 1925].

Discussion.

The demonstration that a carbohydrate complex is one of the structural units present in both the albumin and globulin of horse-serum is important and raises several problems of biological, as well as of chemical, interest. It is somewhat surprising to find that this new constituent is present in relatively large amounts—approximately 2 % of the dry weight of each protein—and one naturally wonders whether any particular biological function is subserved by its presence. Reference will be made later to the work of Glassmann [1925, 1926].

The part which the plasma-proteins play in immunity reactions has been the subject of many researches. In view of the very interesting results which have been obtained recently in a related branch of immunology by Heidelberger and Avery and other workers it is desired to call attention to the fact that the soluble specific substances obtained by these authors from pathogenic bacteria have been shown to be carbohydrate derivatives of a relatively simple structure. Many of these specific substances contain nitrogen, presumably as the amino-group of an amino-sugar.

It is so suggestive to find carbohydrate structures having a complexity at least comparable with that of the carbohydrate present in the serum-proteins and possessed of specific immunological properties, that, whilst avoiding undue emphasis, the present writer would suggest that future investigations concerning the immunological rôle of the plasma-proteins should not overlook the fact that both albumin and globulin contain carbohydrate material as an integral constituent of their molecules.

Returning to purely chemical issues, the question arises of the relationship existing between the serum-proteins and the class of conjugated proteins known as glucoproteins. The work of Levene and La Forge [1913] has shown that the mucins contain a carbohydrate complex which is quite different from the disaccharide complex of the serum-proteins Moreover, the relations existing between the carbohydrate and the serum-proteins are not those usual between prosthetic group and protein moiety; the carbohydrate appears to enter into their structure just as an amino-acid would or as the phosphorus-containing complex enters into the structure of caseinogen [see Rimington, 1927].

Throughout the course of the present research attention has been confined strictly to the two proteins derived from horse-serum. It is hoped, at a later date, to extend and develop the work and, as a preliminary result, it has been ascertained that a carbohydrate substance is present also in wool-keratin. Whether or not it will prove to be the case that carbohydrate complexes are of general occurrence in the protein molecule it is impossible to say, but recently Fränkel and Jellinek [1927] claimed that a carbohydrate composed of glucosamine and mannose occurred in egg-albumin. Unfortunately these authors did not determine the molecular weight of this product, neither did they investigate its structure. Whilst there are points of resemblance between

the disaccharide isolated by me from serum-proteins and the substance obtained by Fränkel and Jellinek, in other characteristics it is notably different. Thus Fränkel and Jellinek state that their preparation was hydrolysed by ptyalin, and furthermore that it yielded no nitrogen gas when treated with nitrous acid in the Van Slyke apparatus. The substance obtained by me from serum-proteins was not attacked by any of the carbohydrase preparations—including ptyalin—which were allowed to act upon it. Its nitrogen, on the other hand, is evolved completely though slowly as nitrogen gas in the Van Slyke apparatus.

Soon after this work was completed Dische [1929] reported some investigations upon the "protein-bound" sugar of the blood. His interest appears to centre round the clinical significance of protein-bound sugar, but by adding phenylhydrazine hydrochloride and sodium acetate to an acid hydrolysate of whole plasma which had been treated with phosphotungstic acid, he succeeded in isolating mannosephenylhydrazone and also in detecting the presence of another, non-fermentable, reducing substance which, he considers, may be glucosamine.

The conditions employed by Dische were such as would hydrolyse not only the plasma-proteins but also the glucosamine-mannose disaccharide which would be obtained, during the course of the hydrolysis, from the albumin and globulin present.

Since, largely owing to the work of Glassmann, the whole subject of protein-bound sugar has recently taken on a new clinical significance, it is of interest to notice how the present research illuminates several long disputed points. Lèpine [1918], to whom the conception of "bound sugar" is due, considered that this was sugar held in glucosidic linkage. Bierry and Fandard [1911], on the other hand, rejected this view since they found that neither emulsin nor invertase increased the reducing power of plasma, boiling acid alone being capable of so doing. The present findings clearly substantiate Bierry and Fandard's views. Secondly, the values obtained by different authors for the proportion of bound sugar have not always agreed. Thus Glassmann [1925, 1926] arrived at figures six or seven times as large as those of Bierry. Dische has shown that alkaline mercuric nitrate—employed as a protein precipitant by Bierry—removes some reducing material from solution. In the light of the present investigations it is clear that the material so lost was glucosamine derived from the glucosamino-mannose.

There remains for discussion one point more; namely, the chemistry of humin formation. Gortner [1916 and elsewhere] has shown that the humin produced from proteins undergoing hydrolysis by boiling acids is derived from an interaction between tryptophan and some substance, most probably of an aldehydic nature, formed together with the amino-acid during the hydrolysis of the protein. Glucose, starch and other carbohydrates were shown to cause an increase in the yield of humin when added along with the protein for hydrolysis. Nothing further is known concerning humin formation,

but the discovery of a carbohydrate complex in two proteins at least, and in relatively large amount, opens up a new field for experimental attack. This carbohydrate may be the precursor of the aldehyde reacting with tryptophan, and the failure of investigators in the past to appreciate its presence is no doubt due in large part to the fact that acid hydrolytic agents were customarily employed in any research upon the nature of the constituent units of the protein. The bulk of the carbohydrate would thereby be lost as a result of secondary reactions.

A systematic examination of these and allied problems is contemplated in the near future. So also is a thorough examination of the different classes of proteins with a view to ascertaining whether the nitrogenous disaccharide described in the above pages is peculiar to the proteins of horse-serum or whether it, or some related compound, occurs universally as a structural unit of protein material.

Summary.

1. From the carefully purified proteins of horse-serum, a carbohydrate derivative has been obtained which is regarded as a definite structural unit of these proteins.

2. The substance isolated has the composition $C_{12}H_{23}O_{10}N$, is devoid of reducing properties and exhibits in solution no measurable optical activity.

3. The constituent sugars have been identified as glucosamine and mannose and it has been shown that the compound has a molecular weight corresponding to that required by the simple disaccharide formula. Identical products are obtained from serum-albumin and globulin respectively and in a yield of approximately 2 % in each case.

4. Calculation from the yield gives figures for the minimal molecular weights of the proteins which are in good agreement with values previously recorded in the literature.

5. Exactly the same product has been obtained from the serum-proteins when trypsin was employed as the hydrolytic agent, thereby establishing the distinction between this carbohydrate and the sulphur-containing tetrasaccharide of the glucoproteins.

6. A structure has been suggested for the carbohydrate substance.

7. Some chemical considerations, arising out of the occurrence of carbohydrate material in these two proteins, have been discussed in connection with physiological and immunological problems.

My thanks are due to the Research Control Committee of this Association for their permission to conclude and publish this research, the greater part of which was carried out at Cambridge. I also wish to thank Sir F. G. Hopkins for his continued interest in this problem and Mr A. Colwell of the Cambridge Biochemical Laboratory for his kindness in carrying out for me a very large number of micro-analytical estimations.

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LII. ON THE NATURE OF THE CARBOHYDRATES FOUND IN THE JERUSALEM ARTICHOKE.

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In spite of repeated investigations, divergencies still exist as to the nature and relative proportions of the carbohydrates found in the tubers of the Jerusalem artichoke (*Helianthus tuberosus*).

The most detailed account of these carbohydrates has been given by Tanret [1893], who carried out investigations with tubers gathered in September and October. Tanret pressed these tubers in a press, collected the juice, and mixed it with about one-tenth its volume of warm basic lead acetate solution of specific gravity 1·25. After filtering off the precipitate formed, the excess lead in the filtrate was removed by means of the requisite amount of dilute sulphuric acid. Addition of hot concentrated baryta water to the clear filtrate thereby obtained yielded a precipitate, which Tanret describes as the barium compound of inulin. Further precipitates could be obtained from this baryta-containing filtrate by addition of increasing quantities of alcohol. Altogether seven carbohydrates were separated from the artichoke juice (see Table I).

Table I.

	Percentage of carbohydrates[1]	
	Calculated on dry tubers	Calculated on total carbohydrates
Inulin	3·12	20·62
Pseudo-inulin	0·35	2 31
Inulenin	1·25	8·26
Helianthenin	0·83	5·49
Synanthrin	7·26	47·98
Sucrose	1·78	11·77
Laevulose and dextrose	0·54	3·57

[1] In his paper Tanret gives his results in grams of carbohydrates per litre of concentrated juice. His figures have been converted as above for the purpose of comparison with the results of other workers.

The reducing sugars laevulose and dextrose were present in small quantities only. The relative proportions of the various other carbohydrates are not given by Tanret, except in the case of a sample of juice pressed from tubers which had been collected in the autumn, but stored in a cellar till the following June before analysis. In consequence of the prolonged storage of these tubers the figures given by Tanret may not be correct; they are quoted, however, as they represent the most complete analysis available in the existing literature.

Other investigators have arrived at different conclusions with regard to the amount of inulin present in artichoke tubers. Thus Konig [quoted by Czapeck, 1913], reports the presence of 58 % of inulin, presumably on the total carbohydrates present; while Shohl [1923] records 15·5 % of inulin in the fresh tubers.

The presence of sucrose in the tubers has been reported by Dubrunfaut [1867] and by Colin [1918]. Helianthenin and synanthrin are names given by Tanret to two laevorotatory carbohydrates with the following characteristics.

Table II.

	Specific rotation $[\alpha]_D^{20°}$	Melting point	Solubility in water	Specific rotation after inversion $[\alpha]_D^{20°}$
Helianthenin	$-23·5°$	176°	50 %	$-70·2°$
Synanthrin	$-17°$	170°	in all proportions	$-70·6°$

Pseudo-inulin and inulenin are closely related to the inulin proper, but are stated to differ from it in their specific rotations, which are said to be for pseudo-inulin $-32·2°$, and for inulenin $-29·6°$. On inversion the carbohydrates show a specific rotation of $-85·6°$ and $-84°$ respectively.

Of all the carbohydrates isolated by Tanret from the artichoke tubers only sucrose and dextrose give a positive specific rotation. It is to be expected, therefore, that the juice obtained by extracting the fresh tubers with water will show a negative rotation. That the juice is laevorotatory is confirmed by Colin [1918], who finds that an extract of the tubers is strongly laevorotatory when prepared in autumn (September). When taken from tubers collected during early spring, Colin obtained dextrorotatory juice. This undoubtedly implies that considerable changes have occurred in the carbohydrates, changes which, according to Colin, result in an altered fermentability of the tubers. Colin points out that it is necessary to subject autumn-gathered tubers to a preliminary hydrolysis with acids before they can be completely fermented by yeast, while the carbohydrates of the tubers collected in spring may be directly converted into alcohol by yeast without preliminary inversion. That a preliminary hydrolysis of the tubers raises the yield of alcohol obtainable from them has also been observed by Rudiger [1920] and by Windisch [1920].

On the assumption that inulin, which is unfermentable by yeast in the pure state[1], is really present in artichoke tubers, the above observations would

[1] An observation which has been experimentally confirmed by the writers.

seem to indicate that inulin undergoes an inversion during storage of the tubers, resulting in its conversion into a fermentable sugar; unless it be considered justifiable to assume with Tanret [1893] that inulin is fermentable by yeast when present in an already fermenting mixture of other carbohydrates. The experiments on which Tanret bases this assumption were carried out with tubers gathered during the early autumn, but stored in a cellar until June of the following year. In the juice obtained from these tubers Tanret found as much as 1·36 % of inulin, calculated on the tubers, of which he was able to recover only 27·5 % after fermentation of the juice by a yeast, 72·5 % of the original amount having disappeared during fermentation.

As certain investigations carried out by the writers made it desirable to collect further information on the fermentative properties of the carbohydrates of the Jerusalem artichoke, it was thought justifiable to repeat part of the work referred to above and to endeavour to co-ordinate the divergent views of previous workers.

As a preliminary it was decided to determine the rotation of juice pressed from tubers collected both during autumn and spring. The results obtained are given in Table III and show that a change from laevo-rotation to dextro-rotation does take place. The actual figures when expressed as specific rotations of the whole mixture of carbohydrates found in the tubers agree closely with the observation of Colin [1918], who observed a specific rotation not exceeding + 10° in tubers stored until Feb.–March. The comparatively strong positive rotation of the carbohydrates in spring juice reaches its maximum about March and has decreased to + 2·12° by May, when germination has proceeded for some time and emptied the tubers of a considerable percentage of their carbohydrates.

Table III.

Date of examination	Percentage of carbohydrates		Observed rotation of juice $[\alpha]_D^{20°}$		Specific rotation of carbohydrate mixture in juice $[\alpha]_D^{20°}$	
	Total	Reducing	Before inversion	After inversion	Before inversion	After inversion
3. xi. 23	3·2	—	−0·095°	−3·13°	−1·48°	−58·9°
16. xii. 23	5·16	0·64	−0·13°	−6·23°	−1·26°	−61·6°
17. i. 24	(a) 3·43 (b) 4·29	(a) *0·31	(a) +0·17°	(b) −4·21°	+1·98°	−61·4°
27. ii. 24	6·19	0·33	+0·71°	−3·58°	+6·06°	−57·84°
18. iii. 24	5·65	0·41	+0·59°	−0·79°	+4·49°	−57·1°
7. v. 24	5·32	—	+0·261°	−0·764°	+2·12°	−58·6°

* Two different juices (*a* and *b*) were used for this determination.

It should be noted that the percentage of reducing sugars in the juice before inversion is very small, even in samples of juice extracted from tubers stored until March. No increase or only a very slight increase in the laevulose and dextrose content has therefore occurred during storage. This is interesting, as it shows that the change in the rotation is not due to an increase in the concentration of a dextrorotatory reducing sugar.

In order to investigate the reason for this change it was decided to attempt the isolation of the various carbohydrates present both in autumn and spring tubers. To do this the method recommended by Tanret was adopted. His method, however, was not found easy to carry out[1], and after several unsuccessful attempts it was decided to proceed with the separation in a somewhat different way, though still adhering to Tanret's method of utilising alcohol of varying strengths for the separation of the individual fractions. The procedure finally adopted was as follows. The freshly-dug tubers were carefully washed and then minced to a fine pulp. The pulp was mixed with its own weight of warm distilled water and the mixture kept at 60°-70° for half an hour. It was then pressed and the juice collected. Basic lead acetate solution of SP.G. 1·25, sufficient to clarify the juice, was added to the extract and the excess lead removed from the clear filtrate with sulphuric acid and sulphuretted hydrogen. The clear filtrate was concentrated *in vacuo* at a temperature not exceeding 42°. Its carbohydrate content was thereby increased to about 20 %. To every 100 cc. of this solution one litre of 95 % ethyl alcohol was added, and the mixture well shaken. The alcohol caused a voluminous precipitate to form when juice obtained from autumn tubers was used. This precipitate was collected on a filter, washed with 95 % alcohol, with ether, and finally dried *in vacuo* over sulphuric acid. When dry it formed a snow-white, light and almost tasteless powder.

For the further purification of this powder it was redissolved in as little warm water as possible, and 95 % ethyl alcohol added to the cooled solution until its alcohol concentration amounted to 50–55 %. The precipitate, after standing overnight, was collected and dried as described above. To the filtrate a further quantity of 95 % alcohol was added to make its concentration equal to about 85 % ethyl alcohol. A precipitate was again formed, which, when dried as already described, formed a granular, sandy-coloured powder. After precipitation of the above two fractions (B and C) the filtrate contained only traces of carbohydrates.

The filtrate from the mixture of clarified juice with alcohol was evaporated *in vacuo* (temperature 42°) to a thick syrup, containing between 40–45 % of carbohydrates. The isolation of these carbohydrates was at first found almost impossible. Finally, however, the following method separated the syrup into two distinct fractions. Three g. of the syrup were poured into 80 g. of absolute alcohol, containing not less than 99·5 % of ethyl alcohol, and the mixture was stirred vigorously for some time. Part of the syrup was thereby converted into a flocculent precipitate, which could be filtered off, while another part formed a gummy substance on the bottom of the container, a third part remaining dissolved in the alcohol. The gum, after dissolution in a very small quantity of water, could again be divided into a flocculent precipitate and

[1] Similar difficulties are also reported by Pringsheim and Lassmann [1922], who were unable to confirm Tanret's results as regards the presence of pseudo-inulin, inulenin, synanthrin and helianthein.

a gum by the addition of the required quantities of absolute alcohol. Repeating this process a sufficient number of times the whole of the syrup could be converted into a flocculent precipitate (fraction D), and a carbohydrate (fraction E) remaining dissolved in this strong alcohol. This latter fraction could be precipitated by the addition of ether to its alcoholic solution, and on separation, formed a gummy and extremely hygroscopic substance, light brown in colour and having a very sweet taste. The flocculent precipitate was not hygroscopic, though extremely soluble in water. It was also light brown in colour and had a sweet taste. The filtrate from the fourth fraction (E) contained traces only of carbohydrates.

The whole of the carbohydrates originally present in the juice had therefore been isolated by the above process.

Subjecting both autumn and spring tubers to the treatment described above the following yields of carbohydrates were obtained (Table IV).

Table IV.

		Juice prepared from tubers dug and extracted Nov. 1923		Juice prepared from tubers dug and extracted March 1924	
Fraction	$[\alpha]_D^{20^\circ}$	Obtained by addition of alcohol	Present as percentage of total carbohydrates	Obtained by addition of alcohol	Present as percentage of total carbohydrates
B	-34.96°	Insoluble in 55 % alcohol	28·04	Insoluble in 55 % alcohol	1·02 (B and C)
C	-33.42°	Insoluble in 85 % alcohol	13·40	Insoluble in 85 % alcohol	
D	Inactive to -1.233°	Insoluble in 98 % alcohol	38·00	Insoluble in 98 % alcohol	44·7
E	-2.91° in autumn to -24.91° in spring	Insoluble in a mixture of ether and 98 % alcohol	20·56	Insoluble in a mixture of ether and 98 % alcohol	54·28

This table is of considerable interest in several respects. It shows, for instance, that there is a very material difference in the distribution of the carbohydrates in autumn and spring tubers, the latter containing less laevorotatory and less insoluble substances than the former. Further, the table appears to explain why artichoke tubers have in some cases been found to contain inulin, while in other cases inulin has been absent.

As seen from Table V fraction B corresponds with the inulin of Tanret; fraction C may be identical with Tanret's pseudo-inulin. These fractions, B and C, disappear from the tubers toward spring and give place to the more readily soluble fractions D and E.

Fractions D and E do not compare with any of Tanret's fractions. They are probably mixtures of carbohydrates, since their specific rotations vary, depending on the season at which they are isolated from the tubers. As both of these fractions yield large percentages of glucose on hydrolysis, the possibility that one of their components is sucrose cannot *a priori* be excluded,

particularly in view of the fact that both Dubrunfaut, Colin and Tanret have isolated sucrose from Jerusalem artichokes.

Table V.

Properties		Inulin	Fraction B	Pseudo-inulin (Tanret)	Fraction C
Specific rotation	Before inversion	$-38°$ to $-39°$	$-34{\cdot}26°$	$-32{\cdot}2°$	$-33{\cdot}12°$
	After	$-85{\cdot}7°$	$-83{\cdot}42°$	$-85{\cdot}6°$	$-77{\cdot}94°$
Reduction of Fehling's solution		No reduction	No reduction	No reduction	No reduction
Solubility in water		Slightly soluble	Slightly soluble, about 0·062 % at 12°	More soluble than inulin	Readily soluble
Melting point		165°–178°	No sharp melting point between 158–163°	Decomposes at 175°	
Effect on ammoniacal silver nitrate		Reduced	Reduced	—	—
Behaviour towards iodine		Yellow coloration	Yellow coloration	—	—
Melting point of osazone after inversion		205°	205°	—	—
Appearance		White flocculent powder, tasteless	White flocculent powder, tasteless	—	Almost white to light fawn-coloured powder with slightly sweet taste

Attempts have repeatedly been made by the writers to isolate sucrose from these fractions D and E, but complete success has not yet been attained. There are good reasons for supposing, however, that fraction E is a mixture of a small proportion of D with a large proportion of sucrose, while fraction D, as isolated by the above treatment, contains a small quantity of sucrose. If it may be assumed that the whole of the dextrose found in the inverted samples of fractions D and E has been derived from sucrose, the following specific rotations would have to be attributed to the remaining components (Table VI, column 4).

Table VI.

Fraction	Specific rotation of fraction calculated from observed rotation	Proportion of dextrose and laevulose in the inverted fraction	Assuming the dextrose found to have been derived from sucrose the remaining compounds of the fractions would have sp. rotation
D from autumn tubers	Zero	22·8 % dextrose 77·4 % laevulose	$-50{\cdot}94°$
D from spring tubers	$-1{\cdot}23°$	23·6 % dextrose 76·4 % laevulose	$-56{\cdot}37°$
E from autumn tubers	$-2{\cdot}91°$	27·9 % dextrose 72·1 % laevulose	$-69{\cdot}0°$
E from spring tubers	$-24{\cdot}91°$	31·7 % dextrose 68·3 % laevulose	$-38{\cdot}3°$

In every case this specific rotation is more negative than that of inulin, probably signifying that the compounds in question are hydration products of inulin, possibly condensation products of laevulose with 6 or 3 laevulose groups, instead of 9 as in the case of inulin [Pringsheim and Aronowsky, 1922].

The question of the fermentability by yeast of the various carbohydrate fractions isolated from the tubers of the Jerusalem artichoke was investigated in a series of experiments the results of which are given in Table VII.

The carbohydrates were dissolved to a concentration of 4 % in a water extract of maize meal, made by digesting 7 parts of maize meal with 100 cc. of physiological saline at 37° for 16 hours in presence of 1 cc. of toluene per litre. The liquid was filtered through an ordinary filter-paper and was then boiled to remove any traces of toluene present[1]. The solutions were sterilised by fractional sterilisation in order to avoid the decomposition of the sugars. After inoculation the samples were incubated at 35° for 4 and 10 days respectively before distillation.

The yeast used in the experiments was a pure culture distillery yeast fermenting well at 35°.

An almost complete conversion into ethyl alcohol was obtained with fraction D and E of both autumn and spring tubers. Fractions B and C, on the other hand, remained unfermented.

Table VII.

Fraction	Carbohydrate concentration of solution to be fermented %	Carbohydrate concentration in mash after fermentation %	Duration of fermentation days	Yield of alcohol obtained calculated on carbohydrates present %	Yield obtained: percentage of that theoretically possible
B	4·2	4·04	10	Traces	—
C	4·2	3·85	10	Traces	—
D autumn tubers	4·2	0·47	4	42·5	88·8
D spring ,,	4·2	0·39	4	43·6	91
E autumn tubers	4·2	0·23	4	44·5	94·6
E spring ,,	4·2	0·23	4	44·5	94·6
Controls—					
Sucrose	4·2	Less than 0·04	4	47·6	99·1
Laevulose	4·2	Less than 0·03	4	49·5	99·3
Dextrose	4·2	Less than 0·03	4	49·5	99·3

The non-fermentability of the B and C fractions agrees well with what would be expected from a carbohydrate of the nature of inulin, though it makes it rather difficult to explain certain earlier experiments and the fermentation experiments of Tanret.

Tanret in his investigations found that 75 % of the inulin present in the tubers used disappeared during the fermentation, and he suggested that inulin might at least be partly fermentable in the presence of readily fermentable

[1] The extract (after inversion) contained less than 0·2 % carbohydrates in solution.

compounds. A number of experiments have been carried out to determine how far this may be the case, and mixtures of two or more of the fractions isolated from autumn-gathered artichoke tubers have been subjected to fermentation by yeast in the manner already described. Table VIII illustrates the results obtained.

Table VIII.

Fraction	Carbohydrate concentration of mash before fermentation %	Carbohydrate concentration of mash after fermentation %	Duration of fermentation days	Yield of alcohol obtained calculated on carbohydrates present %	Yield of alcohol obtained calculated on the fractions respectively B + C %	D %	E %
B + C	4·2	3·99	10	2·7	2·7	—	—
B + C + D	4·2	2·42	10	20·4	2·7	38·12	—
B + C + D + E	4·2	1·51	10	33·0	15·3	43·6	44·5
D	4·2	0·47	4	43·6	—	43·6	—
E	4·2	0·23	4	44·5	—	—	44·5

The fractions were present in the proportions in which they were found in the tubers.

It will be seen that a higher yield of alcohol was obtained in the mash where all four fractions were mixed than should have been obtained had the B + C fraction not been attacked. It is true that the yields of alcohol from the fractions D and E are estimated at 43·6 % and 44·5 % respectively. Yet even assuming that both of these gave yields of 50 %, or the absolute maximum obtainable, the alcohol collected from the mixture of all four fractions would still be in excess of what could be obtained had the B and C fractions remained unattacked.

A second series of experiments entirely confirmed these results. It must be assumed therefore that the B and C fractions can be converted into alcohol to some slight extent when present in a fermenting solution of fractions D and E, though hardly perhaps to the extent stated by Tanret.

The fermentation experiments referred to above as being difficult to reconcile with the presence in autumn tubers of the Jerusalem artichoke of large percentages of inulin were carried out during November 1922 with samples of juice pressed from freshly-dug tubers and fermented with yeast after dilution and sterilisation in the cold by filtration through Chamberland filters. After inoculation with a yeast this cold sterilised juice was incubated at 35° for 6 days. A yield of alcohol was obtained equal to 47·5 % of the total carbohydrates present. As the percentage of carbohydrates present was found to be equal to that of the fresh tubers it is clear that the inulin fractions B and C must have been present in the tubers and, therefore, must have been almost completely fermented under the conditions of the experiment.

Tanret has shown, and the writers have confirmed, that a partial conversion of the inulin fractions into alcohol may occur when these fractions are present in a vigorously fermenting mixture of other carbohydrates, but this partial conversion is hardly sufficient to explain the ease with which practically the whole of the carbohydrates of the cold sterilised juice was found to be converted.

That bacteria, possessing inulin-inverting enzymes, had developed in the cold sterilised fermenting juice is out of the question, since a careful bacteriological analysis proved the juice to be free from all micro-organisms except the yeast used as inoculant. A further possibility, that the sterilisation of the juice had caused the B and C fractions to become inverted, does not arise in this connection, since no heat had been used for the purpose.

One is almost compelled to assume, therefore, that the cold sterilised juice had contained inulase, which remained sufficiently active after filtration to convert the B and C fractions into fermentable compounds during the 6 days during which the juice remained incubated at 35°.

Attempts made to confirm experimentally the assumption of the presence of inulase in the tubers have not met with very great success. In one experiment a mixture of the B and C fractions in the proportions in which they occur in the autumn tubers was added to juice collected from March tubers. It was thought that this juice would contain considerable quantities of inulase and would be capable of inverting the B and C fractions when allowed to act on them under the conditions used in the experiment with cold sterilised juice. After incubation at 35° for 8 days, however, in the presence of toluene to check the development of micro-organisms, only insignificant quantities of the inulin fractions had disappeared. The actual figures in this experiment were as follows: 90·6 % of the inulin fractions were recovered from the incubated test sample of spring juice containing the B and C fractions by the usual procedure of isolation, as against 93·6 % from the control experiment which had not been incubated. A satisfactory explanation of the observations on the fermentability of the cold sterilised juice obtained from autumn-gathered tubers therefore cannot be given.

Table IX.

Sample	Percentages of inulin fractions (B + C) recoverable from mashes autoclaved for 6 hours (steaming)	1 hour at 20 lbs.	2 hours at 20 lbs.	3 hours at 20 lbs.	5 hours at 20 lbs.
Spring juice + B and C fractions in the proportions found in autumn tubers	99·9	90·9	66·8	30	11·4

Table X.

Sample	Yield of alcohol calculated on the carbohydrates present obtainable from samples of inulin containing juice after heating in the autoclave at 20 lbs. for: 6 hours (steaming)	1 hour	2 hours	3 hours	5 hours
Sample juice + B and C fractions in the proportions found in autumn tubers	44·3 %	42·6 %	42·6 %	48·4 %	44·7 %

The control experiment consisting of juice from spring tubers without any inulin fraction added gave a yield of 47·3 % alcohol calculated on the carbohydrates present in the mash.

From a practical point of view this question is not of very great importance. It is of greater importance to decide whether the cooking to which the tubers are usually subjected before fermentation is sufficient to render their inulin fractions fermentable, or whether a special acid hydrolysis of the tubers is essential for this purpose.

A number of experiments have been carried out to investigate this point. Tables IX and X contain the results obtained.

The following figures were actually obtained when calculating the results of the above experiment as the percentage of carbohydrates left after the completion of the fermentation (Table XI).

Table XI.

Sample	Amount of carbohydrates left in the mash after fermentation, and calculated as percentage of the initial carbohydrates present: Steamed for 6 hours	Cooked for 1 hour at 20 lbs.	Cooked for 2 hours at 20 lbs.	Cooked for 3 hours at 20 lbs.	Cooked for 5 hours at 20 lbs.
Spring juice + B and C fractions added in the proportions found in autumn tubers	48·7 %	11·3 %?	22 %	8 %	6·3 %

As a control, the spring juice without addition of the inulin fractions was fermented, and showed 5·7 % of the original carbohydrates remaining after fermentation.

It will be seen that boiling the mashes at a comparatively low pressure for a considerable time is quite sufficient to convert the inulin fractions of the tubers into fermentable carbohydrates, and that an almost full yield of alcohol may be obtained without a preliminary acid hydrolysis of the mashes.

Having ascertained the nature of the carbohydrates found in the tubers of the Jerusalem artichoke it was thought of interest to establish their relationship to the carbohydrates present in the actual parenchymatous tissues of the unripe stalks—the so-called pith. This tissue contains, on an average, 3 % of soluble carbohydrates which can be extracted with warm water. Subjecting this extract to the process used for isolating the carbohydrates of the tubers the following fractions were collected (Tables XII and XIII):

Table XII.

Fraction	Obtained by precipitation: with 55 % alcohol (B)	with 85 % alcohol (C)	with 98 % alcohol (D)	with ether and alcohol (E)
Percentage of total carbohydrates	25·8	4·73	46·27	23·20

The four fractions isolated showed the following properties.

Table XIII.

Fraction	Specific rotation $[\alpha]_D^{20°}$ Before inversion	After inversion	Reduction of Fehling's solution %	Solubility in water	Appearance
B	$-35.4°$	$-85.6°$	1·51	Slightly soluble	Snow-white light powder, tasteless
C	$-30.71°$	$-74.29°$	2·41	Slightly more soluble than B	Slightly yellowish sandy powder, slightly sweet
D	$-17.91°$	$-59.39°$	12·43	Soluble in all proportions of water	Yellow powder, giving brown solutions, very sweet
E	?	?	?	Soluble in all proportions of water	Light yellow gum giving solutions of the same colour, very sweet

It will be seen that not only are the substances isolated from the pith substantially the same as those isolated from the tubers, but they also appear to occur in the pith in about the same proportions as in the tubers.

All fractions isolated from the pith were tested in the same way as those isolated from the tubers and were found to behave similarly.

Summary.

It has been shown that the autumn-gathered tubers and the unripe pith of the Jerusalem artichoke contain considerable proportions of an insoluble non-fermentable carbohydrate, apparently identical with inulin.

This carbohydrate disappears almost completely from the tubers towards spring and gives way to increased proportions of the more soluble carbohydrates which are already present in the autumn tubers. In addition there would appear to occur towards spring an increase in the dextrorotatory compounds of the tubers. Part of these at least is believed to be sucrose.

Though inulin by itself is unfermentable by yeast the experiments quoted in this report would appear to show that a comparatively mild autoclaving is sufficient to render it fermentable without the use of acid hydrolysis.

The observation of Tanret that inulin may be fermented by yeast when present in a solution of already fermenting carbohydrates has been confirmed, though not to the extent claimed by him.

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LIII. THE ACTIVATION OF CERTAIN OXIDASE PREPARATIONS.

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PART I. ACTIVATION BY PEROXIDASES.

1. *LACTARIUS* FUNGI.

SEVERAL instances have been noted in the past of the activation by one means or another of a preparation containing some oxidising enzyme. The best-known instance is that described by Bach and Chodat [1902; 1903, 1, 2] of the activation of the oxidase of the fungus *Lactarius vellereus* by peroxidases. The oxidase was obtained by precipitation of the juice of the fungus with three volumes of alcohol. By repeated precipitation of this oxidase in 40 % alcohol, it was separated into two fractions, neither of which showed much oxidising power apart from the other. The one fraction, insoluble in 40 % alcohol, was very strongly activated by peroxidases, and especially by the other fraction of the oxidase, which was soluble in 40 % alcohol. These authors concluded from their observations that the complete oxidase consisted of two enzymes, (1) an "oxygenase" able to give rise in air to a peroxide utilisable by peroxidase, and (2) peroxidase. On this basis they developed their well-known theory of the constitution of the "direct" oxidases.

In their quantitative experiments, Bach and Chodat used pyrogallol as substrate and measured the activity of their preparations by noting the oxygen absorbed. The experiments were carried out without control of hydrogen ion concentration, and no allowance was made for the autoxidation of pyrogallol. Because of this and the advances in our knowledge of oxidases which have taken place since Bach and Chodat published these experiments, certain possibilities arise which must be considered before the explanation given by them can be accepted.

(1) A change in hydrogen ion concentration by addition of peroxidase preparations with a p_H different from that of the pyrogallol-oxygenase solution might of itself cause an increased activity of the oxidase in *Lactarius*.

(2) Hydrogen peroxide might be produced by the action of an enzyme on pyrogallol, the hydrogen peroxide being normally broken down by catalase, but utilisable instead by peroxidase for oxidation of pyrogallol with consequent increase in oxygen absorption.

(3) There might be oxidation by the action of peroxidase and peroxide of substances in the *Lactarius* preparation which inhibit the enzyme acting on pyrogallol, the peroxide being either (*a*) hydrogen peroxide produced by the

action of the enzyme, or (*b*) a peroxide utilisable by peroxidase and produced by autoxidation of some substance in *L. vellereus*. The possibility of this explanation is based on observations by Bach [1909], who found by estimation of unchanged tyrosine that the action of tyrosinase from *Russula delica* on tyrosine was retarded by peroxidase, even, to some extent, in presence of hydrogen peroxide, although the same end-point was approached. He found, however, that if the tyrosinase preparation were old, then peroxidase with hydrogen peroxide caused initial activation. Bach concluded that substances inhibitory to the action of tyrosinase were produced gradually in the preparation on keeping, these substances being oxidisable by peroxidase with hydrogen peroxide.

(4) The presence in peroxidase preparations of something other than peroxidase itself might be responsible for the increased oxygen absorption. A co-enzyme might facilitate the action of a *Lactarius* enzyme on pyrogallol, and if such co-enzyme were deficient in the *Lactarius* preparations but present in the peroxidase preparation added, activation would be produced which would not be due to peroxidase itself.

(5) With regard to the use of pyrogallol as substrate, Bach and Sbarsky [1911] described the formation from pyrogallol of two types of oxidation product, insoluble purpurogallin, and soluble brownish substances which they believed to be condensation products of purpurogallin. The possibility was therefore to be considered of the directing by peroxidase preparations of the oxidation of pyrogallol in such a way as to increase the amount of oxygen absorbed.

(6) Finally, the "oxygenase" of Bach and Chodat might be interpreted as an autoxidisable substance rather than as an enzyme, and without being necessarily a constituent of all oxidases. That "oxygenase" was such an autoxidisable substance was the conclusion reached by Gallagher [1923] in his investigation of potato "oxygenase."

The phenomenon described by Bach and Chodat [1903, 2] has therefore been more fully investigated with regard to these various points.

Enzymes concerned.

Lactarius vellereus not being available, other *Lactarius* fungi were used in this investigation, namely, *L. blennius*, *L. quietus*, *L. rufus*, and *L. turpis*[1]. The action of appropriate preparations from these fungi on pyrogallol was found to be activated by peroxidase from horseradish, in the manner described by Bach and Chodat for the oxidase of *L. vellereus*, but effective peroxidase preparations from these juices were not readily obtained.

These fungi all contained, as likewise described by Chodat [1910] for *L. vellereus*, both tyrosinase and laccase, but in varying proportions. Guaiacol affords a convenient test for laccase, as it is not oxidised by tyrosinase. Quinol, also, is relatively slowly attacked by tyrosinase. *L. turpis* juice caused much less rapid coloration of buffered guaiacol and quinol solutions than of tyrosine

[1] I am indebted to Dr J. Ramsbottom for kind assistance in identifying some of the fungi.

and *p*-cresol, whereas *L. blennius*, *L. quietus*, and *L. rufus* juices all acted very rapidly on guaiacol and quinol relatively to their action on tyrosine and *p*-cresol. The tyrosinase in these juices could be freed from laccase by alcohol precipitation. A preparation obtained from *L. blennius* juice by two precipitations in 40 % alcohol was found to behave towards phenols as does crude tyrosinase from mealworms, having no action on guaiacol with or without addition of hydrogen peroxide. A similar preparation was obtained from *L. turpis* by 40 % alcohol, and from *L. rufus* in the fraction from 65–80 % alcohol. Tyrosinase appears to be less definitely separated from the juices by alcohol precipitation than is laccase. Fractions obtained from 40–60 % alcohol acted strongly on guaiacol, but were not free from tyrosinase. The two enzymes appear to possess differing susceptibilities to cyanide. The action of *L. quietus* juice on tyrosine was found to be 95 % inhibited by $M/500$ KCN, that of *L. rufus* juice 97 %; whereas Wieland and Sutter [1928] stated that the action on quinol of the enzyme from *L. vellereus* investigated by them was only 67 % inhibited by $M/500$ KCN.

After being heated to 90° for 20 minutes none of the *Lactarius* juices used in the present investigation had any appreciable action on either tyrosine or guaiacol; the thermostable catalyst described by Wieland and Fischer [1926] was therefore not present in any appreciable quantity in any of them. The 40 % alcoholic precipitates all contained some tyrosinase and catalase, and some contained laccase. The "peroxidase" fractions of the fungi prepared as described below all contained tyrosinase, and some contained laccase; the guaiacol-hydrogen peroxide test for peroxidase was positive only with *L. blennius* and *L. turpis* "peroxidase."

Experimental procedure.

Juices of *L. blennius*, *L. quietus*, and *L. turpis* were kept in the ice-chest at a slightly alkaline reaction, and acidified before precipitation to imitate as nearly as possible the reaction of the fresh juices. Some *L. rufus* juice was precipitated fresh, and the remainder was kept in the same way as the other juices. "Oxygenase" fractions were obtained by addition of alcohol to the extent of 40 %, the precipitate being centrifuged, dissolved in slightly alkaline water (residue discarded), acidified, and reprecipitated in 40 % alcohol, as many times as possible with the material available. The first 40 % alcoholic liquid after removal of the precipitate was concentrated *in vacuo*, then alcohol was added to bring it to 65 %, the precipitate centrifuged, and alcohol added to the liquid to bring it to 85 %; this last precipitate was dissolved in water and reprecipitated between the limits about 65–85 % alcohol, as many times as possible, the final solution constituting the "*Lactarius* peroxidase" fraction. Peroxidase from horseradish was prepared by the method described by Bach and Chodat [1903, 1], and, unless otherwise stated, such a preparation is what is meant by the term peroxidase.

The action of "oxygenase" fractions on pyrogallol, and the effect thereon of addition of peroxidases, was investigated by the use of microrespirometers

to measure the rate of oxygen absorption. Throughout the experiments the respirometers were shaken in a water-bath at room temperature, in which they were equilibrated for at least five minutes before the shutting of the taps. Corresponding experiments were shaken side by side, and in such experiments identical buffer solution was used. The following mixtures were placed in the bottles.

Respirometer I. Left: 1 cc. 1·2 % pyrogallol in phosphate buffer p_H 6·0; 0·5 cc. "oxygenase" fraction; buffer to 3 cc. *Right:* 0·5 cc. "oxygenase" fraction; buffer to 3 cc. *Respirometer II. Left:* 1 cc. 1·2 % pyrogallol; 0·5 cc. "oxygenase" fraction; 0·5 cc. peroxidase from horseradish or *Lactarius*; buffer to 3 cc. *Right:* 0·5 cc. "oxygenase" fraction; 0·5 cc. peroxidase; buffer to 3 cc. *Respirometer III. Left:* 1 cc. 1·2 % pyrogallol; buffer to 3 cc. *Right:* 3 cc. buffer. *Respirometer IV. Left:* 1 cc. 1·2 % pyrogallol; 0·5 cc. peroxidase; buffer to 3 cc. *Right:* 0·5 cc. peroxidase; buffer to 3 cc. To each mixture a little phenylurethane or thymol was added as antiseptic, and in the small tube of each bottle was placed excess of strong potash to absorb carbon dioxide. The use of pyrogallol solutions stronger than 1·2 % (2·5 and 5 %) did not increase the rate of oxygen absorption.

Any departures from the above arrangements are mentioned in the text. In calculation of the activation of the "oxygenase" fraction by peroxidases, allowance was made for the effect of the peroxidase preparation used on the autoxidation of pyrogallol, as follows:

$$\text{activation ratio} = \frac{O_2 \text{ absorbed in II} - O_2 \text{ absorbed in IV}}{O_2 \text{ absorbed in I} - O_2 \text{ absorbed in III}}.$$

Ratios above unity indicate activation and below unity inhibition.

Control. The presence of substrate was necessary for the phenomenon of activation to occur. Using *L. quietus* "oxygenase" fraction, in absence of pyrogallol, Respirometer I absorbed over the week-end 19·8 mm.^{3} O_2, II 25·7 mm.^{3} O_2, IV 6·4 mm.^{3} O_2.

Experimental results.

Using "oxygenase" fractions from the fungi named, and peroxidase (*a*) from horseradish, (*b*) from the fungus itself, the following results were obtained:

Fungus	No. of precipitations in 40 % alcohol	Duration of experiment in hours	mm.^{3} O_2 absorbed in respirometers I	II	III	IV	Activation ratio
			(*a*) Horseradish peroxidase				
L. blennius	2	6	32·6	42·5	19	15	2·02
L. quietus (1)	4	6	101	288	18·5	11·7	3·33
,, (2)	4	1	313	356	10·5	8·7	1·15
		3	533	667	21	15	1·27
		6	783	1110	29	22·5	1·44
L. rufus	3	6	371	401	30	23	1·11
L. turpis	2	6	55	58	38	33	1·47
			(*b*) Fungus peroxidase				
L. blennius	2	6	32·6	49·7	19	27·3	1·65
L. quietus	4	6	101	111·5	18·5	29	1
L. rufus	1	6	384	454	30	113	0·96
L. turpis	1	4	44	292	28	268	1·50

In no instances were activations observed as great as those described by Bach and Chodat. This may be due to the phenomenon being better shown by *L. vellereus* than by the varieties of *Lactarius* used in the above experiments.

Most of the horseradish peroxidase preparations were found to retard the autoxidation of pyrogallol, and either to retard the action of the "oxygenase" fraction of *Lactarius* juices on pyrogallol in its early stages, or to produce less activation at the beginning of the experiment than later: figures quoted for *L. quietus* (2) in the first table show this increase in activation ratio with time. The following experiments, in which diluted, unprecipitated juice was used in place of "oxygenase" fraction, and the p_H buffered at 6·5, also show increase in activation with time.

Fungus	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
L. rufus	½	13	10	5	3	0·87
	1	20	21	9	7	1·27
	2	35	40	15	12	1·40
	4	64	83	25	20	1·62
	10	154	224	45	40	1·69
L. quietus	1	120	99	9	11	0·79
	2	206	199	15	17	0·95
	3	287	297	20	23	1·02
	6	475	551	33	37	1·16
	10	629	794	45	53	1·27

The various points raised in connection with the observed activation phenomenon will now be considered in turn, and experiments bearing on them described.

(1) *Possibility of change in hydrogen ion concentration.* Since the solutions were buffered, this effect is minimised. Some of the horseradish peroxidase preparations, which consisted of alcoholic precipitates dried *in vacuo* and dissolved in distilled water, were found to be acid, and the buffering was not able to prevent slight decrease in p_H on addition of such peroxidases. Since Wieland and Sutter [1928] found the optimum p_H for the action of the enzyme from *L. vellereus* investigated by them to be 4·6, using quinol as substrate, a decrease in p_H might of itself activate the *Lactarius* oxidase. The effect of change in p_H on the oxygen uptake accompanying the action of *L. rufus* juice on pyrogallol was therefore examined.

p_H	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	III	I–III
6·0	8	74	27	47
6·5	8	142	34	108
7·0	8	279	242	37

It is shown in the middle column that decrease in p_H did not increase the oxygen taken up by the autoxidation of pyrogallol, and in the last column that, when allowance was made for the autoxidation of pyrogallol, decrease in p_H from 6·5 did not increase the oxygen taken up by the action of enzymes in the juice on pyrogallol. Hence, change in hydrogen ion concentration from a higher to a lower p_H cannot explain the phenomenon of activation.

(2) *Possibility of hydrogen peroxide production* by the action of an enzyme or enzymes in *Lactarius* preparations on pyrogallol. Some catalase was present in all *Lactarius* preparations, so that if hydrogen peroxide were produced and normally broken down by catalase, but on addition of peroxidase utilised for oxidation instead, then there would be an increase in oxygen absorption. This increase, however, would depend on the rate of production of hydrogen peroxide, and in no way could exceed a doubling of the rate. Higher rates than this are here recorded, and the activations observed by Bach and Chodat were even greater. Hydrogen peroxide production in this way, therefore, cannot provide a complete explanation of the phenomenon. The possibility is not precluded that it does take place, and in conjunction with (3), the oxidation of inhibitory substances in *Lactarius* might explain the phenomena.

The work of Wieland and Sutter [1928] furnishes no evidence of the production of hydrogen peroxide by enzymes in *L. vellereus* other than the thermostable catalyst described by Wieland and Fischer [1926]. The former investigators failed to detect any hydrogen peroxide production by their enzyme preparation from *L. vellereus* (apparently a laccase) under conditions in which its production by the Wieland and Fischer catalyst was readily demonstrated.

(3) *Possibility of oxidation of inhibitory substances.* Since the activation ratio increased with time, it seems probable that inhibitory substances either in the *Lactarius* or in the peroxidase preparations added were being oxidised on addition of peroxidase. In this connection the work of Szent-Györgyi [1928] on an inhibitory hexuronic acid oxidisable by hydrogen peroxide in presence of peroxidase and a phenol is of interest.

A consideration against the presence of inhibitory substances in the *Lactarius* preparations is the control experiment described earlier in this paper, which shows that there cannot be present both inhibitory substances oxidisable by peroxidase and peroxide, and substances autoxidisable with production of peroxide. If present along with such autoxidisable substances, they would in any event be oxidised even without addition of peroxidase, by means of peroxidase in the juices. If, however, hydrogen peroxide were derived from the action of an enzyme on pyrogallol, the presence of inhibitory substances in the *Lactarius* preparations, oxidisable by peroxidase + peroxide, might explain the phenomena, since then much greater activation than a doubling in the rate would be possible. They cannot be of the type described by Bach [1909], since, on keeping the *Lactarius* juices under slightly alkaline conditions, their activation by peroxidase did not increase, but eventually disappeared before either tyrosinase or laccase had lost all their activity, tyrosinase being most persistent.

(4) *Possibility of effect not being due to peroxidase itself.* The activation by peroxidase of the action on pyrogallol here discussed appears to be of an entirely different type from the activation of the action of tyrosinase on a mónohydric phenol by boiled tyrosinase preparations, or by *o*-dihydric phenols, discussed later in this paper.

In some qualitative experiments on the liberation of iodine from potassium iodide and the blueing of guaiacum by "oxygenase" with peroxidase, Bach and Chodat [1902] record a control with boiled peroxidase preparation, which did not cause activation. In the present work it was shown that the phenomenon under investigation was likewise not brought about by boiled peroxidase preparation, since, when peroxidase which had been boiled for some time to destroy its activity was used, no activation of the action of *L. rufus* juice was observed.

Duration of experiment in hours	p_H	mm.^{3} O_2 absorbed in respirometers I	II	III	IV	Activation ratio
9	6·5	132	139	37	46	0 98

Use of an amino-acid, glycine, in place of peroxidase preparation did not cause activation of the action of the juice. (Amino-acids are not oxidised by the oxidation products of pyrogallol produced by mealworm tyrosinase. *Respirometer. Left:* 1 cc. 0·2 % pyrogallol in phosphate buffer, p_H 6·0; 1 cc. 0·45 % glycine in buffer; 0·5 cc. mealworm preparation; buffer to 3 cc. *Right:* 1 cc. 0·2 % pyrogallol; 0·5 cc. mealworm preparation; buffer to 3 cc. No change in levels occurred. Tyrosine in place of pyrogallol yielded a similar result, as also did quinol.)

L. rufus juice which had been dialysed to remove any hypothetical dialysable co-enzyme was not more activated by peroxidase than juice which had not been dialysed.

Description of preparation	p_H	Duration of experiment in hours	mm.^{3} O_2 absorbed in respirometers I	II	III	IV	Activation ratio
Undialysed	6 5	8	134	183	20	43	1·23
Dialysed	6·5	8	47	65	29	44	1·17

There was, therefore, no dialysable co-enzyme in the juice, which was supplied by the peroxidase preparations.

(5) *Possibility of change in nature of oxidation products.* Unless excess substrate be present, the rate of action cannot be assumed to be a measure of the activity of the enzyme. When dilute (0·2 %) pyrogallol solutions were used no difference in rates was detectable with or without addition of peroxidase. The oxygen absorption, however, with or without addition of peroxidase, exceeded in a few hours the total oxygen absorption produced by the action of another enzyme preparation, tyrosinase from mealworms, on the quantity of pyrogallol used.

Required for 1 cc. 0·2 % pyrogallol:

calculated value for the production of purpurogallin ...	...	266 mm.^{3} O_2
by action of tyrosinase from mealworms; final figure ...	...	311 ,,
,, *L. quietus* "oxygenase" fraction; 1 day ...	...	448 ,,
,, ,, ,, ,,	+ peroxidase; 1 day	398 ,,

Since the excessive oxygen absorption took place with or without addition of peroxidase, however, it cannot account for the phenomenon of activation[1].

When 2 cc. saturated tyrosine in phosphate buffer p_H 6·0 was used as substrate in place of pyrogallol, activation by peroxidase was still observed, showing that the phenomenon of activation did not depend on choice of substrate.

Fungus	No. of precipitations in 40 % alcohol	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	Activation ratio
L. quietus	4	1	279	267	0·96
		4	403	498	1·24

Wieland and Sutter [1928], using quinol as substrate, tested the action of peroxidase from horseradish on the oxidase preparation obtained by them from *L. vellereus*. The duration of their experiment was 30 min. They found that 4·96 cc. O_2 were absorbed without, and 5·60 cc. O_2 with, addition of peroxidase. These figures they considered scarcely to exceed their experimental error.

(6) *Possibility of the presence of autoxidisable substance in* Lactarius. This appears to be the most likely explanation of the phenomena observed by Bach and Chodat.

Juices which had been exposed to air were, when afterwards tested on pyrogallol, more readily activated by peroxidase than juices which had been kept *in vacuo*, the initial retardation on addition of peroxidase being counterbalanced sooner by the activating action. The following experiments were carried out with *L. quietus* juice. One sample of juice was exposed to air for about one day while another sample stood in an evacuated desiccator. The activation by peroxidase of the action of each sample on pyrogallol was then determined. 1 cc. was used of a peroxidase preparation which had initially a slightly retarding action, and it was only after a number of hours—given in column A of the following table—that the oxygen absorption with peroxidase became equal to that without peroxidase, the oxygen absorption at which this occurred being given in mm.³ in column B. Beyond this point activation set in. It is seen that activation set in more readily when the fungus juice had previously been exposed to air than when it had been *in vacuo*. A 1 and B 1 denote the results obtained with a sample previously exposed to air, and A 2 and B 2 the corresponding results with a sample previously kept *in vacuo*. The whole experiment was repeated three times.

Experiment	A 1 hrs.	B 1 mm.³	A 2 hrs.	B 2 mm.³
1	2½	288	4½	507
2	2	200	4	300
3	5	209	11½	331

A similar effect was observed with the *L. rufus* "oxygenase" fraction. The initial retardation on addition of peroxidase was less when the "oxygenase" preparation had stood for one day than when it was tested immediately after

[1] It appeared to be due to the oxidation of pyrogallol by *Lactarius* giving rise to products different from those produced by the oxidation of pyrogallol by tyrosinase from mealworms.

preparation. It was usually observed also that the initial retardation was slightly greater when 1 cc. peroxidase was used (II b) in place of 0·5 cc. (II a).

Description of "oxygenase" fraction	Duration of experiment in hours	mm.^{3} O_2 absorbed in respirometers		
		I	II a	II b
Fresh	1	92	75	—
	2	162	152	—
One day old	1	97	91	89
	2	163	168	162

These results are consistent with the presence in the juices of a substance which on autoxidation produces a peroxide, and consequently increases the action on pyrogallol when peroxidase is added. There appear also to be inhibitory substances in the peroxidase preparations, which cause initial retardation, and are oxidised away by peroxide with peroxidase.

It follows from the consideration of the experiments so far described that the most likely alternative explanation to the presence of an autoxidisable substance in *Lactarius* juices is the production of hydrogen peroxide by the action on pyrogallol of an enzyme in *Lactarius* precipitated by 40 % alcohol, and the oxidation by peroxidase and this hydrogen peroxide of a further amount of pyrogallol, and also of inhibitory substances in *Lactarius* juices. If this had been the complete explanation, then previous exposure to air of the juices would have been expected to have either no influence on the effect of addition of peroxidase, or, if the inhibitory substances became oxidised in air, to diminish rather than accentuate the observed effect on addition of peroxidase. Since accentuation was observed, the effect was probably due to the alternative explanation, namely, the presence of autoxidisable substance. Although this is evidence of the presence of autoxidisable substance, the production of hydrogen peroxide by the action of an enzyme is not excluded as a partial explanation of the phenomenon.

Further support of the above view was obtained. If by any means the oxidising activity of the juices could be diminished without a corresponding diminution in the degree of activation by peroxidase, then the activation would appear to be a phenomenon not entirely dependent on the action of the enzyme which oxidises pyrogallol. Cyanide was found to affect the action of the enzyme in the juice on pyrogallol much more than it did the process responsible for activation, thus causing a relatively large increase in activation.

Exp. 1. A sample of *L. quietus* juice was tested without cyanide, using 1 cc. peroxidase, and working at p_H 6·5.

	Duration of experiment in hours	mm.^{3} O_2 absorbed in respirometers				Activation ratio
		I	II	III	IV	
Exp. 1	7	675	766	36	29	1·15
Exp. 2	7	156	335	—	—	2·15

Exp. 2. 0·5 cc. $3M/250$ neutralised KCN in buffer p_H 6·5 was added to each bottle of each respirometer, bringing the cyanide concentration to $M/500$, and using otherwise the same materials as in Exp. 1. No alteration in p_H of the

liquids during the experiment was detectable. $M/500$ KCN practically stopped the autoxidation of pyrogallol.

Retardation by cyanide of the action of the juice on pyrogallol was as follows:

Duration of experiment in hours	mm.^{3} O_2 absorbed in respirometers: Without KCN	mm.^{3} O_2 absorbed in respirometers: $M/500$ KCN	Percentage retardation
6	634	132	79

The phenomenon of activation disappeared when the juice had been heated. *L. quietus* juice, which had been heated at 90° for half an hour, gave the following results:

Treatment of juice	Duration of experiment in hours	mm.^{3} O_2 absorbed in respirometers: I	II	III	IV	Activation ratio
			Activation			
Heated	7	64	50	29	29	0·60
			Activity of juice alone			
Unheated	6	634	—	25	—	—
Heated	6	55	—	25	—	—

The loss of activity of the juice itself on heating was 95 %.

Discussion.

The investigation which has been carried out shows that the phenomenon discovered by Bach and Chodat [1903, 2] of the activation by peroxidase of the action on pyrogallol of *Lactarius vellereus* oxidase takes place when certain other *Lactarius* fungi are used. In recent years it has been considered likely that the explanation of the observations which led Bach and Chodat to the formulation of their theory of the constitution of "direct" oxidases was the presence of substances autoxidisable to peroxides. This explanation has been found to be applicable in the present instance. There are in the fungi, in all probability, substances autoxidisable to peroxides utilisable by peroxidase; the addition of peroxidase therefore causes oxidation of further pyrogallol, an effect which is enhanced by removing peroxidase already present in the juices by fractional precipitation with alcohol. It is possible that, besides this, one of the oxidases in the juice may produce hydrogen peroxide when acting on pyrogallol, and may therefore be able to form with peroxidase a system analogous to that discovered in milk by Thurlow [1925], thus providing a partial explanation of the phenomenon of activation by peroxidase. As already pointed out, no thermolabile oxidase in *Lactarius* has been shown to have this function, but it is possible that one may be present able to act in this way, and it is intended to investigate the matter when further material is available. The presence of autoxidisable substance precludes the co-existence in *Lactarius* juices of inhibitory substances oxidisable by peroxidase and peroxide, without which the production of hydrogen peroxide by the action of an enzyme on pyrogallol cannot furnish a complete explanation. The increase in activation ratio with time is probably due to the presence of such inhibitory substances in peroxidase prepared by the method used. The presence of these inhibitory

substances may account for the difference in activating power of peroxidases from different sources observed by Bach and Chodat.

Evidence was not obtained that the oxidases in *Lactarius* juices are separable solely into enzyme-like peroxide and peroxidase, the original view of Bach and Chodat [1903, 2]. A 60-80 % alcohol precipitate from *L. turpis* juice, which was allowed to stand in the ice-chest in contact with 80 % alcohol for 10 days, lost its activity on tyrosine and on guaiacol, although acting slightly on guaiacol after addition of hydrogen peroxide. Since neither the "oxygenase" nor "peroxidase" fraction of *L. vellereus* oxidase obtained by Bach and Chodat had much oxidising power apart from each other, it seems possible that by repeated alcoholic precipitation Bach and Chodat largely destroyed tyrosinase and laccase in both fractions, and separated from one another autoxidisable substance and peroxidase. The view that tyrosinase and laccase are separable into peroxidase and autoxidisable substance does not appear tenable. It seems necessary at present to regard tyrosinase, laccase, and peroxidase with autoxidisable substance as separate distinct systems.

2. Other oxidase preparations.

A. Potatoes. Potatoes were minced and pressed, and the juice was centrifuged; the liquid was precipitated once in 40 % alcohol and the precipitate dissolved as described for *Lactarius* juices. The potato preparations contained tyrosinase, laccase, and catalase. The action of such potato preparations on pyrogallol at p_H 6·5 was not activated by peroxidase, neither was new potato juice unprecipitated by alcohol activated by peroxidase.

Source of preparation	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
Old potatoes	11	138	161	31	46	1·07
Old potatoes	8	132	131	22	54	0·70
New potatoes	11	112	102	31	76	0·32

These results are unexpected in view of the work of Gallagher [1923] on an autoxidisable substance in potatoes. The peroxidase preparations were all neutral; the presence in them of true inhibitory substances is therefore probable. Dialysis of the potato preparations did not result in activation by peroxidase.

Source of preparation	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
Old potatoes	11	101	68	31	46	0·31
New potatoes	11	137	134	31	76	0·55

B. Mealworms. Owing to the absence of laccase, a preparation from mealworms affords convenient material for investigation of the action of peroxidase on the activity of tyrosinase. The preparation contains much catalase, but appears to contain no peroxidase, since it does not act on guaiacol even after addition of hydrogen peroxide, under which conditions a trace of added peroxidase causes immediate coloration of guaiacol. Using the mealworm preparation, prepared according to Raper [1926], filtered, the results (1) were

obtained; dialysed and centrifuged, the results (2). A similar preparation several months old, filtered, was activated (3). The results (4) were obtained by using 2 cc. saturated tyrosine in buffer as substrate in place of pyrogallol, with the mealworm preparation used in (1). The p_H was 6·5, and all peroxidase preparations were neutral.

Description of experiment	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
(1)	10	314	285	23	26	0·89
(2)	7	373	319	31	28	0·85
(3)	10	55	99	23	26	2·28
(4)	8	210	143	—	—	0·68

Thus, only the old preparation was activated by peroxidase. This supports the observations made on *Lactarius* preparations, which indicate that the presence of tyrosinase in the "oxygenase" fractions was not responsible for the activation phenomenon. Since the peroxidase preparations even when neutral had an inhibitory action, the presence in them of inhibitory substances is probable.

The observation recorded by Bach [1909], of the action of peroxidase on tyrosinase, was confirmed qualitatively, using a preparation from mealworms obtained by preliminary extraction with 60–80 % saturated ammonium sulphate, followed by extraction with alkaline water. The darkening of tyrosine by tyrosinase was initially impeded by peroxidase, in presence or absence of hydrogen peroxide, but more in its absence. After one day, all four mixtures were equally dark.

PART II. ACTIVATION OF TYROSINASE.

1. Activation by *o*-dihydric phenols.

It was shown by Raper [1926] that the oxidation of tyrosine by tyrosinase from mealworms was initially activated by 3 : 4-dihydroxyphenylalanine. During the course of the present research, the oxygen absorption accompanying the action of tyrosinase on tyrosine was measured[1]. It was found that the absorption did not start at once, but gradually increased to a constant rate, indicating that the reaction is autocatalytic. This is in agreement with the "catechol" theory of Onslow [1923], as pointed out by McCance [1925]. The addition of dihydroxyphenylalanine or of catechol caused activation by eliminating the initial lag. That this is the interpretation of the phenomenon appears from a recalculation of Raper's figures as rates.

Times (minutes)	0	65	125	205
	Tyrosine oxidised per 20 cc. (mg.)			
Without dihydroxyphenylalanine	0	1·15	2·44	3·54
With dihydroxyphenylalanine	0	1·49	2·54	3·47
Whence, rates per 5 minutes—				
Without dihydroxyphenylalanine	0·088	0·107	0·07	—
With dihydroxyphenylalanine	0·114	0·09	0·06	—

The enzyme used by Raper had not been dialysed.

[1] It amounts to a little over five atoms per molecule of tyrosine; the action on dihydroxyphenylalanine is accompanied by an absorption of a little over four atoms per molecule.

In the present experiments, the following mixtures were placed in respirometers. I. Left: 0·4 mg. tyrosine; 0·5 cc. tyrosinase from mealworms; buffer to 3 cc. Right: 0·5 cc. tyrosinase; buffer to 3 cc. II. Left: 0·4 mg. tyrosine; 0·5 cc. tyrosinase; 0·06 mg. catechol; buffer to 3 cc. Right: 0·5 cc. tyrosinase; 0·06 mg. catechol; buffer to 3 cc. The p_H was 6·0. The oxygen absorbed in mm.³ was as follows:

Rates per 20 min.

I 2; 12; 14; 11; 9; 7; 5.

II 6; 6; 6; 7; 7; 7; 6.

1 cc. saturated tyrosine in buffer was then used as substrate and 0·1 mg. dihydroxyphenylalanine as activator, p_H 7·0:

Rates per 15 min.

I 0; 5; 20; 27; 22; 23; 17; 13.

II 25; 26; 20; 21; 15; 13; 10; 6.

There was no initial lag when, in place of tyrosine, dihydroxyphenylalanine was used as substrate. Using 1 mg. dihydroxyphenylalanine at p_H 6·0:

Rates per 7 min.

I 12; 10; 9; 8; 8.

The enzyme used in the above experiments had been dialysed.

Undiluted tyrosinase preparation acted too rapidly on *p*-cresol for the initial lag to be obvious, but on dilution of the enzyme it became apparent. Using 2 mg. *p*-cresol as substrate, and 0·05 mg. catechol as activator, at p_H 7·0:

Rates per 10 min.

I 3; 9; 10; 13; 13; 12; 11.

II 10; 13; 12; 12; 12; 12; 11.

There was no initial lag when catechol was used as substrate.

Using 2 mg. catechol, at p_H 7·0:

Rates per 5 min.

I 10; 9; 8; 10; 7; 6; 6.

Using enzyme still further diluted:

Rates per 5 min.

I 3·6; 2·9; 3·9; 3·3; 4·2; 2·6; 3·6; 2·9.

The enzyme used in the above experiments had not been dialysed.

In the following experiments the enzyme had been dialysed.

Using 2 mg. *p*-cresol at p_H 7·0:

Rates per hour

I 0; 3; 15.

Using 2 mg. catechol at p_H 7·0:

Rates per 15 min.

I 5; 5; 4.

A similar effect was observed when a *L. turpis* "oxygenase" fraction was used instead of mealworm tyrosinase. Using 2 mg. *p*-cresol as substrate, and 0·06 mg. catechol as activator, at p_H 7·0:

Rates per hour

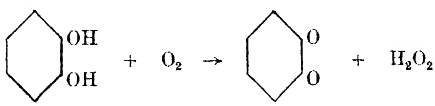
I 0; 0; 0; 19; 26.

II 5; 11; 22; 22; 26.

Discussion.

The phenomena here described have a significant bearing on the mode of action of tyrosinase. Since the action on the monohydric phenols tyrosine and *p*-cresol is autocatalytic, and the lag is eliminated by addition of an *o*-dihydric phenol, it appears as if some product of action on an *o*-dihydric phenol enabled the enzyme to act on the monohydric phenol. Since dialysis of the enzyme preparation did not destroy its action on the monohydric phenol, the necessary product appears not to be dialysable. It is quite likely, however, that in some instances the requisite *o*-dihydric phenol may be present as an impurity in the monohydric phenol, a possibility included by Onslow [1923]. Some old samples behaved towards peroxidase and hydrogen peroxide as if they contained some corresponding *o*-dihydric phenol, and such samples showed no initial lag when tyrosinase acted on them. This possibility is under further investigation, although it cannot furnish a complete explanation, as follows from the work of Onslow and Robinson [1928] described below.

The production of hydrogen peroxide by the action of tyrosinase has not so far been demonstrated, and if the preparation from *L. vellereus* used by Wieland and Sutter [1928] contained tyrosinase, then the production of hydrogen peroxide by tyrosinase is unlikely. The production of *ortho*-quinones has been demonstrated [Happold and Raper, 1925; Pugh and Raper, 1927]. If, nevertheless, the reaction



expresses what occurs when tyrosinase acts on an *o*-dihydric phenol, in accordance with the suggestion of Onslow and Robinson [1926], then both *ortho*-quinone and hydrogen peroxide are possible activators of the action on a monohydric phenol. Onslow and Robinson [1928] believe the effective substance to be an *ortho*-quinone. Using an enzyme obtained from the potato by preliminary treatment with alcohol, followed by aqueous extraction of the enzyme, they obtained, by means of treatment with charcoal, preparations which acted strongly on catechol, but scarcely at all on *p*-cresol or tyrosine. Addition of a trace of catechol caused action on *p*-cresol, and slight action on tyrosine. They concluded from their experiments that the oxidation of the monohydric phenols is a secondary phenomenon depending on the presence of an *ortho*-quinone and not directly on the action of the enzyme. They assumed that alcohol did not extract the *ortho*-quinone from plant tissue, and that it was an *ortho*-quinone which was removed by charcoal.

An *ortho*-quinone has not been shown to convert a monohydric to an *o*-dihydric phenol. Also it seems unlikely that an *ortho*-quinone is the effective substance, because dihydroxyphenylalanine will serve as activator, although the *ortho*-quinone formed from it is not "free" (as shown by its inability to oxidise an external amino-acid), but condenses at once with another part of its own molecule [Raper, 1927]. The following considerations indicate that

neither *ortho*-quinone nor hydrogen peroxide is able to bring about the conversion of a monohydric to an *o*-dihydric phenol at the requisite rate (the rate of a series of reactions being determined by the rate of the slowest), unless possibly it is catalysed in some way. Peroxidase with hydrogen peroxide, as well as tyrosinase, produces *ortho*-quinones from *o*-dihydric phenols such as catechol [Pugh and Raper, 1927], yet peroxidase with hydrogen peroxide does not act on tyrosine, even in presence of a trace of added catechol, although peroxidase with hydrogen peroxide is known to act rapidly on dihydroxyphenylalanine, the *o*-dihydric phenol produced from tyrosine by tyrosinase [Raper, 1926], to give products indistinguishable from those produced by tyrosinase. Neither does peroxidase with hydrogen peroxide act on phenol. It follows, since peroxidase with hydrogen peroxide is able to carry out all further stages in the reactions, that neither hydrogen peroxide nor *ortho*-quinone is able to convert monohydric to *o*-dihydric phenol, unless the change is catalysed by something specifically present in tyrosinase preparations. Until enzymes catalysing action on monohydric and *o*-dihydric phenols have been shown to be distinct from one another, the name "tyrosinase" must be applied to both, since action on tyrosine by means of a co-enzyme is a distinctive property of the enzyme.

The facts may possibly indicate that the conversion of monohydric to *o*-dihydric phenol is brought about by hydrogen peroxide (Dakin's reaction), catalysed by tyrosinase, possibly by way of an oxidised form of the enzyme; but further work is required before the mechanism responsible for action on a monohydric phenol can be known.

2. Activation by boiled preparations.

The observation was made by Haehn [1919], and confirmed by Raper and Wormall [1923], that the action of tyrosinase from potatoes on tyrosine was activated by boiled potato juice. This has been confirmed for tyrosinase prepared from mealworms.

Recalculation of Raper and Wormall's figures in terms of rates shows that the phenomenon consists in a tendency to elimination of initial lag.

	Tyrosine present in g. per 330 cc.				
Ordinary potato juice	0·1427	0·1269	0·1062	0·0905	0·0321
Potato juice + boiled potato juice	0·1442	0·1185	0·0980	0·0804	0·0287
Time (min.)	0	130	220	360	1380

Whence rates per 10 min. (mg.)—

Without boiled juice	1·21; 2·30; 1·12; 0·57.
With boiled juice	1·98; 2·28; 1·26; 0·51.

Using the arrangement of respirometers described for activation by *o*-dihydric phenols, 1 cc. saturated tyrosine as substrate, and 1 cc. boiled mealworm preparation as activator, at p_H 7·0:

	Rates per 15 min.
I	0; 5; 20; 27; 22; 23; 17; 13.
II	25; 30; 23; 21; 14; 12; 7; 7.

The boiled preparation had been dialysed before boiling.

That activation by boiled preparations is related to the activation by *o*-dihydric phenols does not appear unlikely.

SUMMARY.

1. The phenomenon discovered by Bach and Chodat of the apparent activation by peroxidases of a constituent of the oxidase of the fungus *Lactarius vellereus* separable from it by 40 % alcohol is considered.

2. Using peroxidase from horseradish, the phenomenon has been confirmed for some other *Lactarius* fungi, and certain alternative explanations to that put forward by Bach and Chodat are discussed.

3. The most probable interpretation of the phenomenon is the presence in *Lactarius* fungi of an autoxidisable substance. The possible presence also of some enzyme able to produce hydrogen peroxide by dehydrogenation of the substrate may contribute to the phenomenon. No evidence was obtained of the dual constitution ascribed by Bach and Chodat to oxidases in general.

4. The effect of peroxidase from horseradish on the action of potato juice and of tyrosinase from mealworms is described.

5. The action of tyrosinase on tyrosine and on *p*-cresol is shown to be autocatalytic, the lag being eliminated by addition of a small amount of catechol or of dihydroxyphenylalanine.

6. The suggestion put forward by Onslow and Robinson [1928], that an *ortho*-quinone is responsible for initiation of action on a monohydric phenol, is discussed.

7. The elimination of lag by boiled tyrosinase preparations is demonstrated.

I wish to express my thanks to Professor H. S. Raper, under whose direction this work was carried out, for his kind criticism and help throughout the investigation.

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LIV. SOME ENZYMES IN *B. COLI COMMUNIS* WHICH ACT ON FUMARIC ACID.

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It was shown by Quastel and Woolf [1926] that when fumaric acid and ammonia are incubated with a suspension of *B. coli communis* at p_H 7·4 in presence of an "inhibitor," such as 2 % propyl alcohol, there is a disappearance of free ammonia from the solution. Similarly, when *l*-aspartic acid is incubated under the same conditions, it is partially deaminated, and the time-course and final values of the ammonia uptake or output correspond with those required for the reversible reaction

$$COOH.CH=CH.COOH+NH_3 \rightleftharpoons COOH.CH_2.CH(NH_2).COOH$$

where the molecular equilibrium constant $K \doteq \frac{[\text{Fumaric acid}]\,[\text{Ammonia}]}{[\text{Aspartic acid}]}$ has a value of about 0·04. In the absence of "inhibitors," there is also, under anaerobic conditions, an irreversible complete deamination of the aspartic acid, with production of succinic acid. It was shown by Cook and Woolf [1928] that the mechanism responsible for this reductive deamination is independent of the enzyme governing the aspartic-fumaric equilibrium, since it is present in strict aerobes and strict anaerobes, which do not bring about the latter reaction. When equilibrium has been attained, both aspartic acid and fumaric acid can be isolated, but, while the yields of aspartic acid agree satisfactorily with the theoretical, the yields of fumaric acid are invariably very low.

The presence in animal tissues of an enzyme capable of forming malic acid from fumaric acid was shown by Batelli and Stern [1911], who named it "fumarase." Einbeck [1919] reported that the reaction stopped when about three-quarters of the fumaric acid had been converted, and Dakin [1922] confirmed this and proved that the malic acid produced was exclusively the *laevo*-form. Clutterbuck [1927] followed polarimetrically the production of *l*-malic acid from fumaric acid in presence of muscle, and found that the reaction followed a linear course until the equilibrium was nearly reached, when it slowed down. Alwall [1929] completed the proof that a true chemical equilibrium was involved by showing that the same final state was reached when muscle tissue was allowed to act on *l*-malic acid.

The presence of fumarase in *B. coli* was reported by Quastel and Whetham [1924]. It is obvious that if this enzyme were present in the bacillus in any

considerable quantity it would interfere with the aspartic-fumaric equilibrium, giving a complex malic-fumaric-aspartic equilibrium as the final state. Quastel and Whetham, however, found that when fumaric acid was incubated anaerobically with relatively large concentrations of organisms, only minute quantities of malic acid could be detected. They therefore concluded that there was only a small quantity of fumarase in *B. coli*, and in the previous work on the aspartic-fumaric equilibrium this conclusion was accepted, and it was taken for granted that the production of malic acid was so slow as not to interfere appreciably with the main reaction. It will be shown in this paper that the interpretation given by Quastel and Whetham of their results was erroneous, though justified by the facts known at the time they did their work; that, in fact, *B. coli* shows a very high fumarase activity; and that the equilibrium constant previously reported for the aspartic-fumaric equilibrium is really that of the complex malic-fumaric-aspartic equilibrium. It will also be shown that malic acid itself does not take up ammonia, that the addition of water and of ammonia to fumaric acid is due to two distinct enzymes, and that it is possible to eliminate the fumaric-malic reaction and so obtain the true constant of the equilibrium between aspartic acid, fumaric acid and ammonia. The bearing of these results on the "active centre" hypothesis of Quastel and Wooldridge will be discussed.

Experimental.

All the work described was done with suspensions of "resting" *B. coli communis*. The organism was grown, either in Roux bottles containing 150 cc. of Cole and Onslow's tryptic broth, or on the surface of tryptic broth agar in Petri dishes. There was no apparent difference in the behaviour of the organisms obtained by the two methods. In each case, the nutrient medium was inoculated from an 18 hours old broth culture, and incubated at 37° for 2 days. When agar plates were used, the growth was washed off with normal saline and centrifuged; when broth was used, this was centrifuged from the organisms. The deposit of *B. coli* was then washed three times by centrifuging in normal saline, and finally suspended in saline and aerated for a few hours. It was stored at 0°, and, although generally used fresh, did not lose its activity after several months. The growth from one Roux bottle or one Petri dish generally corresponded to about 10 cc. of the suspension.

The following stock solutions were used: $M/2$ sodium fumarate, $M/2$ sodium *l*-aspartate, $M/2$ sodium *l*-malate, and M ammonium chloride. The organic acids were weighed out and neutralised with sodium hydroxide, and all solutions were brought to p_H 7·4. The buffer used was Clark and Lubs's phosphate buffer, p_H 7·4, containing $M/20$ phosphate. Ammonia was estimated on 0·5 cc. samples by the method of Woolf [1928]. Malic acid was estimated polarimetrically as the molybdate compound, by the method of Auerbach and Kruger [1923]. A 5 cc. sample was added to 10 cc. of 14·2 % ammonium molybdate, then 1 cc. of glacial acetic acid was added, and the mixture was

allowed to stand a few hours in the dark. It was then filtered through kieselguhr, exactly 5 cc. of water being used for wetting the filter-paper and washing, so that the total volume of fluid used was 21 cc. The presence of the molybdate helped to precipitate the bacteria, so that a crystal-clear filtrate was obtained. Another 5 cc. sample was added to 11 cc. of 6 % trichloroacetic acid, which was filtered in the same way with the addition of 5 cc. of water. The trichloroacetic acid acted as a protein precipitant and produced about the same degree of acidity as that due to the acetic acid in the molybdate mixture. The two solutions were examined polarimetrically in a 2 dm. tube, and the difference in rotation was proportional to the *l*-malic acid present. It was found that 10 mg. of malic acid, under these conditions, gave a rotation difference, with light from the mercury green line, of + 0·84°. This agrees with the value given by Needham [1927]. All the reaction mixtures were incubated at 37°, the mixtures without inhibitor being contained in filter-flasks evacuated at the water-pump, while those with inhibitor were placed in stoppered flasks, it having been previously ascertained that there was no difference in the course of the reaction anaerobically and aerobically.

RESULTS.

The result of a typical experiment demonstrating the fumarase activity of the organism is shown in Fig. 1. The following reaction mixtures were made up, each containing in addition 50 cc. buffer solution and 2 cc. of *B. coli* suspension.

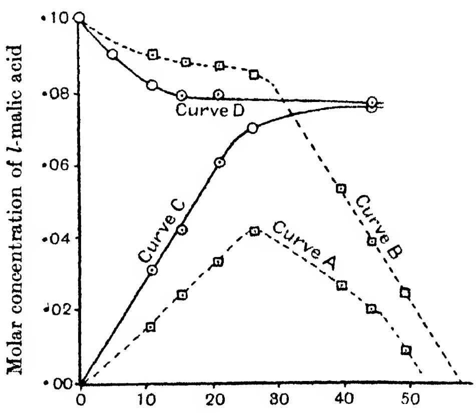

Fig. 1.

Mixture	M/2 fumaric cc.	M/2 *l*-malic cc.	Propyl alcohol cc.	Water cc.
A	20	—	—	28
B	—	20	—	28
C	20	—	2	26
D	—	20	2	26

Initial malic acid estimations were made, the mixtures were incubated, *A* and *B* being anaerobic, and at suitable intervals further samples were taken for estimation.

Curves *C* and *D* show that in presence of 2 % propyl alcohol an equilibrium is reached when about 76 % of the fumaric acid is converted into malic acid, or 24 % of the malic to fumaric. This equilibrium value agrees with that found by many workers for muscle. The initial portion of curve *C* is linear, as found by Clutterbuck [1927, 1928] for the fumarase of muscle and liver, which suggests that the enzyme is saturated with its substrate for nearly the whole course of the reaction. Curves *A* and *B* show that in the absence of an inhibitor the fumaric-malic equilibrium is masked by some other irreversible reaction which results in the destruction of the malic acid, and follows a linear course. The final portion of curve *A* is invariably parallel to the linear part of curve *B*, but sometimes the curves cross before this part is reached. The same phenomenon is observed in the absence of inhibitors for the aspartic-fumaric

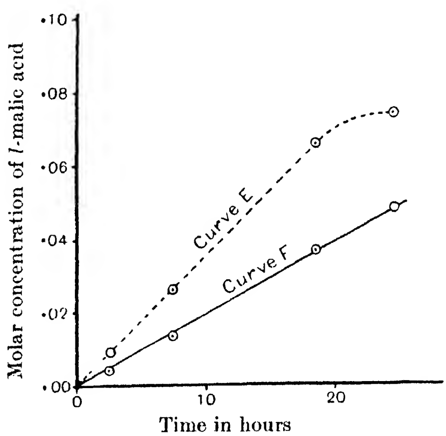

Fig. 2.

equilibrium [cf. Quastel and Woolf, 1926, figs. 1 and 2; Cook and Woolf, 1928, fig. 1], and, as in these cases, the chemical change involved is no doubt a reduction to succinic acid of at any rate part of the malic acid. The matter is under investigation. The figure also shows that the presence of propyl alcohol increases the velocity of the fumarase action. This activation is not always observed.

It was shown by Clutterbuck [1928] that phosphates had an activating action on animal fumarase. This has been confirmed for the enzyme of *B. coli*, as shown by Fig. 2. Curve *E* was obtained with a mixture similar in composition to mixture *C* of Fig. 1, and for curve *F* the buffer was replaced by water. The figure shows that the presence of $M/40$ phosphate at p_H 7·4 almost doubles the reaction velocity. All the other reaction mixtures used in this work contained half their volume of buffer solution.

It is now possible to explain the results of Quastel and Whetham [1924]. Their reaction mixture was similar to that used for curve *A*, save that they used a much higher concentration of *B. coli*. When they examined their solution, at the end of 24 hours' incubation *in vacuo*, it had probably reached the final part of curve *A*, and most of the malic acid previously formed had disappeared, thus leading them to believe that there was very little fumarase in the organism.

Having established the presence in *B. coli* of considerable quantities of fumarase, it became necessary to re-examine the supposed aspartic-fumaric equilibrium. A typical result is shown in Figs. 3 and 4. The reaction mixtures were:

Mixture	*M*/2 *l*-aspartic cc.	*M*/2 fumaric cc.	*M* NH_4Cl cc.	Propyl alcohol cc.	Water cc.
G	20	—	—	2	26
H	—	20	10	2	16
K	—	20	—	2	26

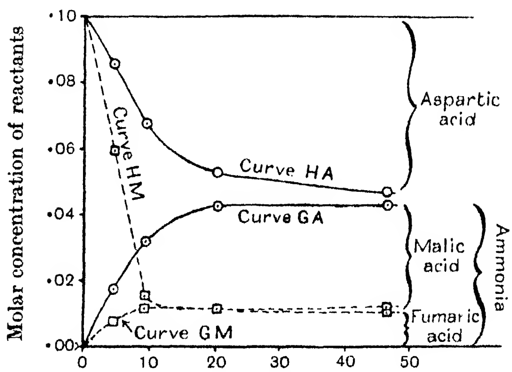

Fig. 3.

Ammonia and malic acid estimations were made on *G* and *H* and malic acid estimations on *K*; the solutions were then incubated and the estimations were repeated at suitable intervals. The results with *G* and *H* are shown in Fig. 3. Curves *GA* and *GM* represent the changes that take place when *M*/10 aspartic acid is incubated with the organism. The distance between *GA* and the base-line represents the molar concentration of free ammonia, found by estimation. This will of course be equal to the sum of the concentrations of fumaric and malic acids. The distance between curves *GA* and *GM* represents the concentration of malic acid, as found by estimation. Hence, by difference, the distance between *GM* and the base-line gives the amount of fumaric acid present, while the aspartic acid concentration is equal to the distance between *GA* and the horizontal line at the top of the figure. Similarly, for the other pair of curves, *HA* denoting free ammonia in *H* and the distance between *HA* and *HM* the malic acid. It will be seen that the ammonia curves *GA* and *HA* are similar

to those given by Quastel and Woolf [1926], but that, at equilibrium, the fumaric acid concentration is only about a quarter of the ammonia concentration, the remaining three-quarters having changed to malic acid, in accordance with the requirements of the fumaric-malic equilibrium.

Fig. 4 is strong evidence that the addition of water and of ammonia to fumaric acid is the work of separate enzymes. It is conceivable that the two reactions could be effected by a single enzyme, which activates fumaric acid, possibly on the lines suggested by Quastel [1926], the activated fumaric acid then "accepting" either water or ammonia, in the form of their ions, which do not need "activation" (Quastel). If this were the case, and one started with a system containing the enzyme and fumaric acid only, then the rate at which malic acid was formed would be governed by the speed with which the

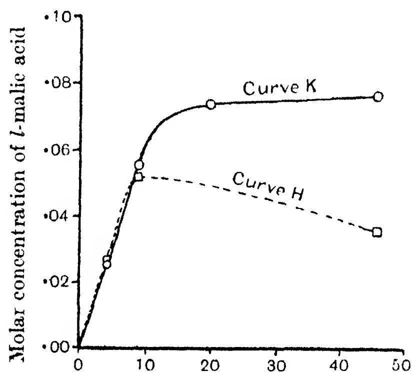

Fig. 4.

enzyme could activate fumaric acid. Now, if ammonia were added to the system, some of the fumaric acid which would have yielded malic acid would be diverted to form aspartic acid; there would be competition between water and ammonia for the activated fumaric acid. On the other hand, if two distinct enzymes were involved, then, so long as the fumaric acid was present in sufficient concentration to saturate the fumarase, the addition of ammonia would not affect the rate of formation of malic acid. Fig. 4 shows that this is in fact the case. Curve H shows the rate of malic acid formation from $M/10$ fumaric acid in presence of $M/10$ ammonia, *i.e.* the ordinate at any time equals the distance between curves HA and HM in Fig. 3. Curve K shows the production of malic acid in mixture K, which contains no ammonia, and it is clear that the initial rates of malic acid production in the two mixtures are identical.

When malic acid and ammonia are incubated with *B. coli* the final state is the same as that shown in Fig. 3, but the ammonia uptake is slower than with fumaric acid. It is, of course, possible that malic acid goes directly to aspartic acid without passing through the stage of fumaric acid. That this is unlikely is shown by Fig. 5. Mixture L contains $M/10$ fumaric acid and $M/10$

ammonia, together with a very small quantity of organism, while in mixture *M*, malic acid is substituted for the fumaric acid. The curves show the details of the beginnings of the ammonia uptakes. Fumaric acid immediately begins to react, but with malic acid there is a lag, indicating that fumaric acid must be formed before the ammonia uptake can begin.

The equilibrium constant K_1 for the reaction

$$COOH.CH=CH.COOH + H_2O \rightleftharpoons COOH.CH_2CH.OH.COOH$$

is given by the equation

$$K_1 = \frac{[\text{Fumaric acid}]\,[\text{Water}]}{[\text{Malic acid}]}.$$

The concentration of water can be taken as constant. Hence the ratio of fumaric acid to malic acid at equilibrium will be constant, whatever their initial concentrations may be. Now in the aspartic-fumaric equilibrium the constant K_2 is given by

$$K_2 = \frac{[\text{Fumaric acid}]\,[\text{Ammonia}]}{[\text{Aspartic acid}]}.$$

The value found for this constant by Quastel and Woolf [1926] was 0·04, but what they supposed was the fumaric acid concentration was really the sum of the fumaric and malic acid concentrations. The true concentration of fumaric acid is only a quarter of the value they used. It is this constancy of the

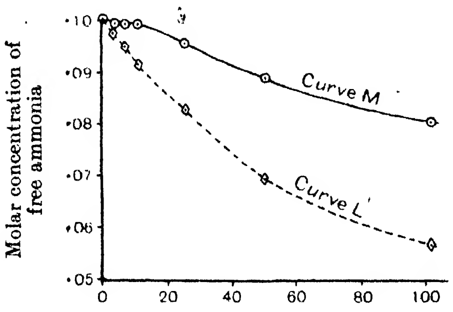

Fig. 5.

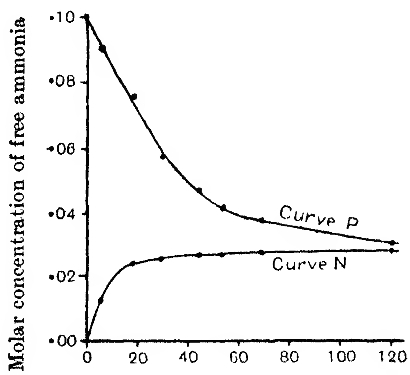

Fig. 6.

fumaric-malic ratio that enabled them to find the same value of K for the complex equilibrium when they varied the concentrations of their reactants. Substituting the true value of the fumaric acid concentration in the equation for K_2, one finds that K_2 is about 0·01. From this value it can easily be calculated that, if the fumaric-malic reaction could be entirely eliminated, the equilibrium mixture for the aspartic-fumaric equilibrium, when the reactants were present in the usual initial concentrations of $M/10$, would contain only about 28 % of free ammonia instead of the 47 % found for the mixed equilibrium.

Fig. 6 shows the result of an experiment in which these conditions are realised. The effect of cyclohexanol on the fumarase of *B. coli* is peculiar. If

the organism is incubated with 2 % cyclohexanol for an hour or two, the fumarase is markedly activated, but if the incubation is continued for 15 hours, the fumarase activity is sometimes found to have entirely disappeared. This is not always the case, and it is probable that the time required for the destruction of the enzyme varies for different strains and preparations of the organism. When the fumarase is destroyed in this way it is found that malic acid will show no ammonia uptake, but ammonia uptake by fumaric acid still occurs. In the experiment to be described, 20 cc. of stock *B. coli* suspension were added to 78 cc. of saline and 2 cc. of cyclohexanol, and the mixture was incubated for 15 hours, when the fumarase was found to be completely destroyed. The following reaction mixtures were then made up:

Mixture	*M*/2 *l*-aspartic cc.	*M*/2 fumaric cc.	*M* NH_4Cl cc.	Buffer cc.	Cyclohexanol cc.	Treated *B. coli* cc.	Water cc.
N	20	—	—	50	1·8	10	18·2
P	—	20	10	50	1·8	10	8·2

The mixtures were incubated, and ammonia and malic acid estimations made at intervals. The malic acid estimations were negative, and the ammonia results are shown in Fig. 6. It will be seen that the final value obtained is the true one for the aspartic-fumaric equilibrium.

This differential destruction of the mechanisms responsible for the two reactions is a further proof that two distinct enzymes are involved, and the absence of ammonia uptake by malic acid after the destruction of fumarase confirms the conclusion that malic acid itself is not converted into aspartic acid. It is convenient to have a name for the enzyme responsible for the aspartic acid reaction and it is proposed that it be provisionally termed *aspartase*.

It was found by Alwall [1928] that the fumarase in a succinoxidase preparation from muscle is destroyed by incubation at 50° for half an hour. Boiled *B. coli* shows no fumarase or aspartase activity, and preliminary experiments indicate that the inactivation temperature for each enzyme is in the neighbourhood of 50°.

Discussion.

B. coli communis is able to bring about several chemical reactions involving fumaric acid. One is the irreversible reaction shown in Fig. 1, which is inhibited by propyl alcohol. Possibly another is the oxidation of fumaric acid, though it seems probable that this may only take place *via* malic acid. Finally there are three reversible reactions: the fumaric-succinic-methylene blue equilibrium [Quastel and Whetham, 1924], and the equilibria governed by fumarase and aspartase. Each of these reactions seems to be catalysed by a distinct enzyme, which can effect one reaction only. Working with mammalian tissues, Alwall [1928] destroyed fumarase and left succinoxidase, and Clutterbuck [1928] destroyed succinoxidase and left fumarase. It has been shown in this paper that the fumarase of bacteria can be destroyed without elimination of aspartase. Quastel and Wooldridge [1927, 1] showed that the succinoxidase of

B. coli is destroyed by incubation with cyclohexanol for 5 minutes, whereas fumarase has been shown above to be far more resistant to this substance. They also showed that succinoxidase is little affected by one hour at 57°, a treatment that would destroy both fumarase and aspartase. Quastel and Wooldridge [1928] report that, after treatment with toluene, the affinity of the succinoxidase of *B. coli* for malonic acid is vastly increased, so that small concentrations have a large effect in retarding the reaction velocity. Experiments made on fumarase and aspartase show that they do not possess this property. Finally, repeated attempts have been made to demonstrate the presence of aspartase in muscle, with uniform lack of success, although the various preparations used showed marked fumarase activity.

It seems clear, therefore, that three reactions as similar as the addition across the double bond of fumaric acid of 2H, H and OH, and H and NH_2, require separate enzymes for their catalysis. Now according to the "activation" theory of Quastel [1926], as modified and extended by Quastel and Wooldridge [1927, 2] into the "active centre" theory, one would expect the essential happening in all these reactions to be the same, the activation of fumaric acid. The fumaric acid would be adsorbed or combined at an active centre, at which it would come under the influence of an intense electric field, becoming activated, so that its state could be diagrammatically represented as $COOH.CH_2.\check{C}.COOH$. The activated molecule would then react with ions from the solution, which do not need activation, and which would combine at the temporarily unsaturated or active carbon atom represented by the sign $\check{C}$. If this were the true mechanism, one would expect all three reactions to be effected by a single enzyme; or, if it were objected that the three reactions required fumaric acid activated to different extents, then it would be anticipated that the enzyme that could effect the most difficult of the three reactions —the one requiring the highest energy of activation or the most intense electric field—should also be able to bring about the other two reactions, while the enzyme catalysing the more difficult of these two reactions should also bring about the easier, and only the enzyme with the weakest field should be truly specific for one reaction only. But experiment shows that the contrary is the case; each enzyme is specific for one type of reaction. The differences in the enzymes seem not to be merely quantitative, as one would expect if the views of Quastel and Wooldridge were accepted as adequate, but there appear to be qualitative differences between them.

At the present stage it is only possible to give the merest indications of what the nature of these qualitative differences may prove to be. It will be noticed in curve *HA*, Fig. 3, that the initial portion is linear for a large portion of the reaction. This suggests that the enzyme is working at full saturation with its substrate. Now the reaction taking place involves two substances, fumaric acid and ammonia. If it were supposed that the ammonia were acting in solution, combining with activated molecules of fumaric acid at the enzyme when it came into contact with them by collision, then, by the law of mass

action, it would be expected that, as the ammonia concentration fell, the reaction velocity would fall proportionately, even although the enzyme was saturated the whole time with fumaric acid. One would only expect to get a linear reaction if the enzyme were saturated with both the reactants—fumaric acid and ammonia. It follows that one must suppose that ammonia is combined at the enzyme as well as fumaric acid. Similar considerations apply to the reduction of methylene blue by succinic acid in presence of bacteria. Quastel [1926] gives a figure (Fig. 1) showing that the rate of decoloration is linear during the greater part of the reaction. This implies that the enzyme is saturated with methylene blue, and therefore that there is combination between enzyme and dye. Further evidence leading to the same conclusion is furnished by the work of Dixon [1926] on xanthine oxidase and Quastel and Wooldridge [1927, 1] on several of the dehydrogenating enzymes of *B. coli*. These workers used various dyes of different reduction potentials, and found that the rate of reaction was not dependent merely upon the ease of reduction of the indicator. Dixon points out that the presence of sulphonic groups in the dye tends to slow its reduction rate. These facts suggest that the chemical nature of the dye affects its affinity for the enzyme, and hence its rate of reaction. Quastel and Wooldridge themselves state that "the velocity of reduction is governed by the concentration of indicator as well as by the concentration of activated donator at the surface," but they do not seem to mean that the combination between enzyme and indicator is of the Michaelis type, nor do they seem to the writer to bear this condition sufficiently in mind in the development of their theory.

These considerations, and similar ones applied to the facts known about some other enzymes, point to the rather attractive hypothesis that one of the conditions for a reaction to occur at these enzymes is that all the substrates shall be combined there together—succinic acid and methylene blue for succinoxidase action, fumaric acid and ammonia with aspartase, and fumaric acid and water with fumarase. Whether there is also activation by electric fields as Quastel postulates, or whether the mechanism by which reaction is effected is of a different nature, seems to the writer still a very open question. This hypothesis has at any rate the merit that it is capable of being tested on strictly quantitative lines, and it is hoped to carry out further studies on fumarase and aspartase with this object in view.

SUMMARY.

1. The presence is demonstrated in *B. coli communis* of considerable quantities of fumarase, the enzyme governing the equilibrium between fumaric acid and *l*-malic acid. In the absence of inhibitors, such as 2 % propyl alcohol, the action of this enzyme is masked by an irreversible process resulting in the anaerobic destruction of malic acid. In presence of propyl alcohol the same equilibrium is attained as that given by animal fumarases.

2. The equilibrium previously reported between aspartic acid, fumaric acid and ammonia is really a complex malic-fumaric-aspartic equilibrium. The fumarase of *B. coli* can be destroyed by treatment of the organism with cyclohexanol, and then the true aspartic-fumaric equilibrium is obtained.

3. The three reversible changes involving fumaric acid—those to succinic, malic and aspartic acids—are catalysed by distinct enzymes. It is proposed to call the enzyme responsible for the third of these reactions *aspartase*.

4. The bearing of these results on enzyme theory is discussed, and a limited hypothesis on enzyme action is put forward.

It is a pleasure to express my gratitude to Sir F. G. Hopkins and Mr J. B. S. Haldane for their continued encouragement during the course of this work.

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LV. THE DISTRIBUTION OF VITAMIN B_2 IN CERTAIN FOODS.

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By vitamin B_2 is here meant the factor in the vitamin B complex, other than the antineuritic, which promotes growth and cures and prevents dermatitis in rats. Its possible identity with the "P. P." factor, preventive and curative of human pellagra, and probably of "black-tongue" in dogs, is discussed later in this paper.

Since the discovery that McCollum and Davis's [1915, 1, 2] water-soluble B vitamin can be subdivided into at least two factors, Eijkman's [1897] antineuritic and another [Smith and Hendrick, 1926; Goldberger *et al.*, 1926; Chick and Roscoe, 1927], no attempt has been made to work out systematically the distribution of the more heat-stable factor, vitamin B_2. The distribution of the other constituent, the antineuritic vitamin B_1, as investigated by the power of a food to protect a bird on polished rice from polyneuritis or to cure the condition when it is developed, is presumably not affected by the discovery of another factor in the vitamin B complex. As early as 1912 Cooper [1912] showed that pigeons require to maintain their weight on a diet of polished rice a factor different in its distribution from that which protects against and cures polyneuritis, and it is possible that this factor was what is here designated vitamin B_2. But, since autoclaved yeast, one of the richest sources of vitamin B_2, has no effect in curing polyneuritis, it would follow that the presence or absence of vitamin B_2 in the food does not affect its strictly antineuritic potency. The distribution of the thermolabile antineuritic factor B_1, in so far as it has been investigated by experiments with birds fed on polished rice, has therefore not been upset by later discoveries.

Where the "water-soluble vitamin B" value of a substance has been investigated with the growth of rats as a criterion, it is not possible, since both factors are equally necessary for growth, to form any idea of the relative amounts of vitamins B_1 and B_2 present. Chick and Roscoe's [1928] work showed that it is difficult to clear caseinogen completely of vitamin B_2, and it may reasonably be assumed that most "vitamin B-free" diets have hitherto not been free of this factor. Moreover, it is not until recently that techniques have been evolved for supplying a rat with vitamin B_1 without vitamin B_2.

Distribution of vitamin B_2 as indicated by previous observations on the growth of rats.

In the course of previous work demonstrating the dual nature of vitamin B, some indications of the distribution of vitamin B_2 have been given. This factor is abundant in yeast, fresh or autoclaved [Smith and Hendrick, 1926; Chick and Roscoe, 1927; Sherman and Axtmayer, 1927]; 0·2 g. daily of fresh yeast and 0·4 g. of autoclaved maintained growth in rats receiving daily an antineuritic yeast extract free from vitamin B_2 [Chick and Roscoe, 1927]. Sherman and Axtmayer [1927] found when observing growth in rats on a vitamin B-free diet that whole wheat supplemented autoclaved yeast, and dried skim milk supplemented whole wheat, and drew the conclusion that whole wheat is richer in vitamin B_1 (F) than in vitamin B_2 (G), while with milk the reverse is the case. Hunt and Kraus [1928] found that 5–10 cc. of fresh whole milk daily supplemented, as source of "vitamin B," a yeast-fraction which could also be supplemented by autoclaved yeast. A number of observations on pigeons have also indicated that milk is a poor source of the antineuritic vitamin [Cooper, 1914; Gibson and Conception, 1916; Johnson and Hooper, 1921].

Chick and Roscoe [1927] observed that it required a daily dose of 1 g. of wheat embryo to produce normal growth in conjunction with an antineuritic concentrate prepared according to Peters's method [Peters, 1924; Kinnersley and Peters, 1925], whereas 0·2 g. daily of the embryo could produce good growth when supplemented by autoclaved yeast.

McCollum, Simmonds and Pitz [1917] state, without giving experimental results, that the antineuritic value of the oat kernel is high; by analogy with other cereals one would expect this to be the case. Smith and Hendrick [1926], however, found that a diet containing 40 % of whole oats failed to produce normal growth in rats unless supplemented by 5 % fresh or autoclaved yeast, suggesting that oatmeal is richer in vitamin B_1 than in vitamin B_2.

Salmon [1927] obtained results suggesting that the leaves of the velvet bean were richer in vitamin B_2 than the beans themselves, while the distribution of vitamin B_1 was the opposite.

Goldberger *et al.* [1926] reported that rats fed on a diet containing 20 % of dried fresh beef as the sole source of vitamin B rapidly declined and died, with or without polyneuritis. 5 % of an alcoholic maize extract, which, supplemented by autoclaved yeast, could produce good growth, but of which alone as much as 40 % produced no growth, was added. With this addition the rats grew in a normal manner, suggesting that dried beef is a stronger source of vitamin B_2 than of vitamin B_1.

The results of these investigations can be roughly summarised as follows. Unheated yeast is rich in both vitamins B_1 and B_2; autoclaved yeast only in vitamin B_2. The cereals contain more vitamin B_1 than vitamin B_2, milk and meat the reverse. In the velvet bean the leaves are richer in vitamin B_2, the seeds in vitamin B_1. It is impossible to make any quantitative estimates of vitamin B_2 from these observations.

EXPERIMENTAL.

In the present research a thorough investigation of the wheat and maize kernels for their vitamin B_2 content was made; the point of interest being to determine, since human pellagra is a maize, rather than a wheat, eater's disease, any significant difference in vitamin B_2 content of the two cereals. We have also investigated certain other common foodstuffs in a less exhaustive fashion.

Method used for assaying vitamin B_2.

The technique elaborated by Chick and Roscoe [1928] was used. In this method B_1 is supplied to the rats as Peters's antineuritic concentrate, while special efforts are taken to free the caseinogen in the basal diet from vitamin B_2 by thorough washing with acidulated water and extraction with dilute acid alcohol. It is difficult to check all growth in rats and induce pellagra-like symptoms in them unless this method of purification is followed.

Our basal diet P_2L was as follows:

Specially purified caseinogen	20	parts
Rice starch	60	,,
Cotton-seed oil	15	,,
Salt mixture[1]	5	,,
(Water	100	,,)

The diet was cooked in a steamer for 3 hours at 100° to prevent refection [Fridericia *et al.* 1927; Roscoe, 1927].

Diet P_2L, with the daily addition of 0·05–0·1 g. of cod-liver oil to provide vitamins A and D, and 0·1 cc. (equivalent to 0·6 g. yeast) daily of Peters's antineuritic concentrate [Peters, 1924; Kinnersley and Peters, 1925], to provide vitamin B_1, was assumed to supply all the factors necessary for rat growth except vitamin B_2 (or the part of the vitamin B complex other than the antineuritic).

In the investigation of the cereals the materials under examination were mixed into the basal diet in such proportions that the standard ratio between protein, carbohydrate, and fat (20 : 60 : 15) was not altered, using for the calculation the commonly accepted analyses of the cereals. For example, white flour roughly contains: protein 10 %, carbohydrate 90 %; in constructing a diet containing 65 % of white flour, the following proportions were accordingly employed:

Purified caseinogen	13·5	parts	
Rice starch ...	1·5	,,	
Cotton-seed oil ...	15·0	,,	
Salt mixture ...	5·0	,,	
White flour ...	65·0	,,	(6·5 parts protein; 58·5 parts carbohydrate)
(Water ...	100·0	,,)	

making the percentages of protein, etc., similar to those in the basal diet P_2L. The composition of other cereal diets was similarly calculated. The diets were cooked for 3 hours at 100°. We did not in any case allow the cereal protein to replace more than one-half the caseinogen of the original basal diet; in most cases it replaced about one-third.

In the investigation of other foods, of which the necessary quantities were small, daily additions were made to the basal diet P_2L.

Young rats, just weaned, from 30 to 45 g. in weight, were fed for a week on diet P_2L, with the addition of three drops (0·05 g.) of cod-liver oil daily to provide vitamins A and D, Peters's antineuritic concentrate being omitted for economy's sake. At the end of the week, during which growth always ceased, the rats were put in separate cages and the diet containing the substance under examination was fed to them, 0·1 cc. (=0·6 g. dry yeast) daily of Peters's antineuritic concentrate (vitamin B_1) being now added. One rat from each litter, usually the best grown, was kept on diet P_2L with additions of cod-liver oil and antineuritic concentrate only, to act as a "negative control."

[1] Salt mixture No. 185 [McCollum, Simmonds and Pitz, 1917].

Young rats, when first caged singly and put on a strange diet, take a few days to get used to new conditions. Very little growth may take place in the first week, even on a good diet. We therefore disregarded the first week's growth, and took for our standard of comparison the average weekly growth during the succeeding 4 weeks, the vitamin B_2 value of a foodstuff being estimated by the minimum daily ration needed to maintain normal increase in body-weight (11–14 g. weekly) during those 4 weeks. In a few cases when, after a test period of 2 to 3 weeks, it became obvious that the diet contained insufficient vitamin B_2 for growth, we increased the amount of test substance in the ration and began again, using the succeeding 4 weeks for our standard of comparison.

We found sometimes a different response to the same diet by rats from different litters. This difference was not large enough to produce markedly incompatible results, but made it necessary to distribute each test material through several litters, so that in no case should conclusions be drawn from results obtained on rats of one litter only. Males and females were used in approximately equal numbers in testing each foodstuff.

Where it seemed of interest to compare as accurately as possible the vitamin B_2 content of certain foods, such as "household" and "top patent" flour and whole wheat and whole maize, rats from the same litter were used, as these give a more uniform response.

The power of certain foodstuffs to cure dermatitis which had developed in rats on the basal diet was also tested (Table V).

1. *The vitamin B_2 content of various samples of wheat, maize and peas, tested as different proportions of the diet.*

Two kinds of whole wheat were tested, (A) a mixture consisting mainly of Manitoba wheat, (B) a sample of English wheat. Of sample (A) five different parts of the berry were tested, viz. (1) the germ, which in this sample appeared to be quite free of bran, (2) the bran, (3) "top patent" flour, a sample specially collected and consisting of almost pure endosperm, (4) "household" flour, a coarser article than the "top patent" and one that is in commercial use, and (5) "tails" or fine pollard, a mixture of all parts of the berry carrying a high percentage of bran. The last was tested with the idea that it might represent a more or less pure specimen of the aleurone layer which lies between the bran and the endosperm, but on further investigation the sample was found to be too mixed for such conclusions to be drawn.

Two samples of whole maize were tested, (A) white African maize, (B) yellow South American maize. Maize germ meal and maize "grits" from sample (B) were also investigated. The maize germ meal contained a fair amount of endosperm, the maize "grits" was, as far as was ascertainable, fairly pure maize endosperm. "Polenta," another form of maize endosperm, was also tested.

In view of the extreme importance of the comparison of whole wheat and whole maize, an additional experiment was set up in which the two samples of whole wheat and the two samples of whole maize were tested on rats of similar weights from the same litter. The result of this experiment is shown in Fig. 1.

A sample of dried peas was tested.

The "polenta" and the peas were bought across the counter, the other samples being obtained direct from the mills.

The results of these experiments are shown in Table I, and in Table II are given the minimal amounts which must be present in the diet if normal growth is to be maintained.

Table I. *Average weekly growth increments of rats on:* A, *a mixed diet;* B, *purified diets, deficient in vitamin B_2, and with this vitamin supplied by autoclaved yeast;* C, *synthetic diets in which vitamin B_2 is supplied by various percentages of cereal products and peas.*

(Vitamin B_1 supplied in B and C by daily doses of Peters's antineuritic concentrate from yeast.)

	Material tested	No. of rats observed	Average weekly increase in body-weight for chosen 4 weeks (g.)
A	Mixed diet. Dried or fresh winter milk, white or brown bread, cabbages or carrots. With Quaker oats, wheat bran, sheep's lungs and cods' heads once or twice a week	16 stock rats from 5th–9th week of life	14 ♂ 16; ♀ 12.
B	Negative controls. Diet P_2L	15	2
	Positive controls. Diet P_2L + autoclaved yeast (0·4 g. daily)	5	12·5
C	Wheat. Whole ground wheat. Sample A, Manitoba, 30 %	2	5·5
	50 %	10	16
	Sample B, English 50 %	4	12
	Wheat germ. Sample A, 7·5 %	4	2·5
	15 %	6	9
	30 %	6	16
	Wheat bran. Sample A, 15 %	4	8
	30 %	4	13·5
	Wheat "tails" (fine pollard). Sample A, 15 %	4	7·5
	30 %	4	15
	Flour. Top patent. Sample A, 65 %	7	5
	Household. Sample A, 65 %	4	5
	Maize. Whole ground maize. Sample A, white, African, 30 %	4	8·5
	50 %	6	11
	Sample B, yellow, S. American, 50 %	8	8
	Maize germ meal. Sample B, 15 %	4	2
	30 %	3	7
	60 %	6	13
	Endosperm. Maize "grits." Sample B, 65 %	6	5
	"Polenta," Italian, 65 %	4	4
	Peas. Dried ground "Clipper" peas, 30 %	2	9·5
	45 %	5	16

Whole wheat cannot be regarded as a rich source of vitamin B_2, since 30 % in the diet of sample A, a mixed sample consisting chiefly of Manitoba wheat, proved insufficient for growth; with 50 % of this wheat in the diet excellent growth was obtained. The sample of English wheat (B) was not as good a source of the vitamin; with 50 % in the diet growth was only just normal.

Wheat endosperm, either as "top patent" flour or as the less pure "household" flour, is a poor source of vitamin B_2, 65 % of either produced very little growth. Wheat embryo and bran are about equal to each other in their vitamin B_2 potency, and to wheat "tails"; in each case 15–30 % provides sufficient vitamin for normal growth. Tibbles [1912] gives the composition of the wheat berry as follows: bran 13·5 %, endosperm 85·0 %, germ 1·5 %, so that, in the wheat kernel as a whole the bran is the most important source of vitamin B_2.

Table II. *Minimum amounts of cereal products, given incorporated in the diet, required to provide enough vitamin B_2 for normal growth in young rats (11–14 g. weekly increase in body-weight).*

Material	Amount required % of diet
Wheat. B, English. Whole grain	50
A, Manitoba. Whole grain	30–50
Embryo	15–30
Bran	15–30
Pollard	15–30
Patent flour	>65
Household flour	>65
Maize. A, white, African. Whole grain	50
B, yellow, S. American. Whole grain	>50
Germ meal	30–60
Endosperm "grits"	>65
C, endosperm Italian "polenta"	>65
Peas. Dried "Clippers"	30–45

N.B. Where two figures are given the lower was insufficient.

The mixed wheat sample, Manitoba predominating, was typical of the wheat mixtures used in London flour mills. It must be remembered, however, that since our investigation was made on particular samples, it is impossible to assume that these are representative.

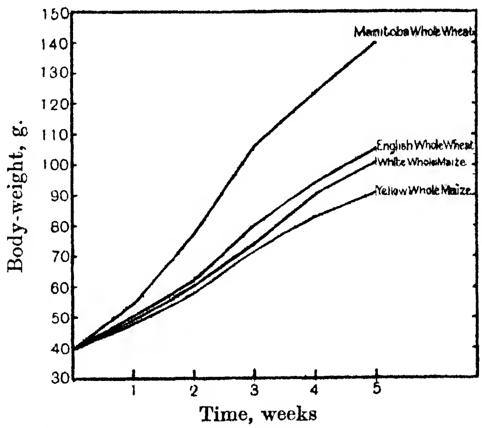

Fig. 1. Comparison of growth-promoting value of two samples of whole maize and two of whole wheat, when given as the only source of vitamin B_2 in an otherwise complete diet. The curves show the average growth of two rats (1 ♂ and 1 ♀) on each diet. 50 % of the cereal product was included in the diet in each case.

Whole yellow maize is definitely inferior in its vitamin B_2 content to either of the samples of whole wheat tested. With 50 % in the diet the rats did not grow normally. The sample of white maize tested had a higher vitamin B_2 value than the yellow sample. The same amount, 50 %, in the diet was needed to produce normal growth as was needed of English wheat; it was, therefore, much inferior to the mixed Manitoba wheat (see Fig. 1).

Maize endosperm, as "grits" or as "polenta," is very low in the vitamin, as is wheat endosperm; with 65 % in the diet the rats scarcely grew at all. The maize germ meal used was a less cleanly milled product than the wheat germ. In the maize kernel the germ is attached to the endosperm by a large

scutellum and can only be prepared in the pure state by careful hand dissection. Maize germ meal, therefore, carries of necessity a large proportion of both endosperm and scutellum. Even taking this into account, the maize germ may be considered a poorer source of vitamin B_2 than wheat germ, as 60 % was needed to produce a normal rate of growth as against 30 % of wheat germ.

Vitamin B_2 and the antineuritic vitamin are differently distributed in the wheat and maize kernels. Vitamin B_2 is not entirely absent from the endosperm as is vitamin B_1. The rats on the 65 % white flour, and 65 % maize "grits," diets grew slightly better than those on the basal diet and did not develop pellagra-like symptoms except in one case, when a rat on 65 % "top patent" flour developed mild dermatitis. As regards the antineuritic vitamin B_1 "the germ, weight for weight, was found to be five times as potent as the bran in the cure of polyneuritis" [Medical Research Council, 1924]. In contrast with this, the vitamin B_2 content of the bran and the germ are about equal.

The vitamin B_2 value of dried peas cannot be considered high, 45 % in the diet being necessary before normal growth was obtained.

2. *The vitamin B_2 content of various foodstuffs, tested as additions to the basal diet.*

Yeast, dried and autoclaved for 5 hours at 120°, fresh whole winter milk from the ordinary London supply, cooked dried egg yolk, dried beef steak and dried ox liver were tested for their vitamin B_2 content by feeding small doses daily to rats which received the basal diet with the addition of Peters's yeast concentrate as source of vitamin B_1.

Table III. *Average weekly growth increments of rats on a purified caseinogen diet deficient in vitamin B_2 (P_2L), supplemented with daily doses of various foods.*

(Vitamin B_1 supplied by daily doses of Peters's antineuritic concentrate from yeast.)

Material tested	Amount given daily: Air dry g.	Amount given daily: Natural state	No. of rats observed	Average weekly increase in body-weight for chosen 4 weeks (g.)
Liver (dried ox liver)	0·06	0·2 g.	2	4
	0·12	0·4	3	12
	0·25	0·8	1	18·5
Yeast (dry)	0·1	0·5 g.	2	8
	0·2	1·0	3	16
Yeast (autoclaved)	0·4	2·0 g.	5	12·5
Milk (fresh whole, London winter supply)	0·36 solids	3 cc.	3	9
	0·72 solids	6	3	12
	0·96 solids	8	2	14
Egg yolk (cooked, dried)	0·5	1 g.	2	7
	1·0	2	4	11
Meat (dried steak)	0·25	13	2	3·5
	0·5	2	3	9
	0·75	3	3	13
	1·0	4	2	15

The results of this experiment are given in Table III, and in Table IV are shown the minimum amounts which must be given if normal growth is to be maintained.

Table IV. *Minimum amounts of substances containing vitamin B_2 given as daily additions to a basal diet (P_2L), required for the normal growth of young rats (11–14 g. weekly increase in body-weight).*

Material	Daily dry weight g.	Daily amount as natural foodstuff
Liver (dried ox liver)	0·06–0·12	0·2–0·4 g.
Yeast (dried)	0·1 –0·2	0·5–1·0 g.
Yeast (autoclaved)	0·4	2·0 g.
Milk (fresh whole, London winter supply)	0·36–0·72	3–6 cc.
Egg yolk (cooked, dried)	0·5 –1·0	1–2 g.
Meat (dried steak)	0·5 –0·75	2–3 g.

N.B. Where two figures are given the lower was insufficient.

All these natural foodstuffs enabled rats to increase normally in weight when given in doses of 1 g. and under.

Since we kept a daily record of the food consumption of each animal, it is possible to draw rough comparisons between the foods which were incorporated as different percentages of the diet and those fed as additions to it. Rats growing at a normal rate on diet P_2L (with additions) consumed from 6 to 7 g. (dry weight) daily. A dose of 1·0 g. dry weight would therefore represent 14–12·5 % of the dry weight of the total food consumed, one of 0·5 g. 7–6 %. Normal growth was obtained on the cereal diets only when 30 % or more of the cereal product was included in the total dry weight consumed, or when 2 g. and upwards of the cereal product was consumed daily.

The substances tested in Table III are therefore to be reckoned better sources of vitamin B_2 than the cereal products and peas, if the comparison is made on the dry weights of the substances concerned.

The following rough comparison of the foodstuffs tested may therefore be made, the substances being placed in descending order as regards their B_2 content, this being reckoned on the dry weight.

1. Dried ox liver
2. Dried yeast
3. Autoclaved yeast
4. Meat (dried steak)
5. Milk solids
6. Dried egg yolk
7. Wheat germ
8. ,, bran
9. ,, pollard
10. Dried peas
11. Whole wheat (Manitoba)
12. ,, ,, (English)
13. Whole maize (white)
14. ,, ,, (yellow)
15. Maize germ meal
16. Wheat endosperm
17. Maize endosperm

3. *The curative effect of various foodstuffs on the dermatitis and other symptoms developing in rats on a vitamin B_2-deficient diet.*

Of 15 rats from 15 different litters kept as negative controls on diet P_2L with the addition only of cod-liver oil and antineuritic concentrate, none showed significant growth, and 9 developed symmetrical dermatitis. Of these 8 displayed lesions of the fore-paws, the hind feet also being affected in 6 cases and the groins and axillae in 3; one rat showed dermatitis of the groins and axillae alone. The axillary lesions tended to spread down the inner side of the fore-limbs and across the chest and those in the groins down the inner side of the thighs. The dermatitis of the extremities was usually accompanied by oedema, which in 2 rats was so severe as to cause gangrene by constriction, with the loss of six toes in one case. The general condition of the rats showing skin symptoms was usually miserable.

The average time taken for symptoms to appear was 10 weeks, the shortest period being 5 weeks, and the longest 22.

The remaining 6 rats deprived of vitamin B_2, which showed no localised dermatitis, developed nevertheless a condition of general wretchedness after a period varying from 5 to 8 weeks. They showed a mixture of such symptoms as ophthalmia, inflamed nostrils, loss of hair on head and elsewhere, and haematuria. These symptoms were equally frequent in the rats showing definite dermatitis.

The symptomatology was as described in a previous paper from this laboratory [Chick and Roscoe, 1928], except that skin symptoms developed more constantly. A curious feature was the tendency of certain symptoms to predominate at certain times. At one period in the autumn the sick rats tended to develop severe lesions of the extremities and of the eyes, while later the groins and axillae were most often affected, the eyes being almost normal.

The power of certain foodstuffs to cure these symptoms of dermatitis was tested on eight of these negative control rats and on four other similar rats, negative controls from other experiments.

In addition to these young rats, two adult rats, aged about 1 year, were put on the vitamin B_2-free diet. One of these (801 ♀) dropped in weight from 350 to 170 g. in 11 weeks, and died without showing skin symptoms, though developing some ophthalmia. The other (802 ♂) dropped from 200 to 140 g. in 10 weeks, its weight then became stationary and at the end of 12 weeks it began to show dermatitis, which rapidly became very severe. This rat was used for a curative experiment.

The results of the curative experiments are given in Table V.

In these experiments it was found that the growth and healing properties of the substances tested ran parallel. The foodstuffs were mostly the same as those of which the vitamin B_2 content had been tested by growth experiments. The results confirm the idea that in the rat a growth-promoting and a dermatitis-preventing factor are identical. In addition to the substances used in the

Table V. *The curative effect of various substances on the dermatitis and other symptoms developing in rats on a vitamin B_2-deficient diet (P_2L).*

Rat	Condition before test	Substance tested	Length of time substance was fed	Result
771 ♀	Ophthalmia. Wretched condition. Weight stationary	Cheddar cheese 1 g. daily	3 weeks	Very slight improvement; 9 g. increase in wt.
714 ♂	Severe ophthalmia. Wretched condition. Dermatitis of extremities. Growth stationary	Cheddar cheese 2 g. daily	3 weeks	All lesions healed; 28 g. increase in wt.
711 ♀	Poor general condition. Dermatitis of axillae, groins and feet. Cessation of growth	Fresh meat 0·4 g. daily (0·1 g. dry wt.)	2 weeks	Improvement in dermatitis; 5 g. increase in wt.
734 ♀	Severe dermatitis of axillae and groins. Poor general condition. Cessation of growth, loss of weight	Cooked liver 0·4 g. daily (0·12 g. dry wt.)	3 weeks	Complete cure; 37 g. increase in wt.
3061 ♂	Severe dermatitis of fore-paws. Ophthalmia. Nose inflamed. Haematuria. Cessation of growth	Wheat germ 7½ %	1 week	No improvement or resumption of growth
		Wheat germ 15 %	1 week	Ditto
		Wheat bran 15 %	1 week	Ditto
		P_2L + 1·0 cc. yeast fraction ≡ 5 g. dry yeast	2 weeks	Complete cure; 28 g. increase in wt.
3067 ♂	Dermatitis of fore-paws. Inflamed nose. Haematuria. Cessation of growth	Patent flour	5 days	Died
378 ♂	Severe ophthalmia. Diarrhoea. Cessation of growth	Patent flour 65 %	3 weeks	Temporary improvement, followed by regression; no growth
		P_2L + 0·4 g. autoclaved yeast	3 weeks	Complete cure; 47 g. increase in wt.
728 ♂	Dermatitis of tail and extremities. Commencing gangrene of two toes. Cessation of growth	Maize germ meal 60 %	3 weeks	Toes sloughed off and all dermatitis healed; 30 g. increase in wt.
748 ♂	Severe dermatitis of fore-paws and feet. Commencing gangrene of toes. Dermatitis of tail. Weight stationary	Maize germ meal 60 %	3 weeks	Complete cure of dermatitis; toes sloughed off and stumps practically healed; 31 g. increase in wt.
729 ♂	Severe dermatitis of fore-paws. Weight stationary	Milk 6 cc. daily	3 weeks	All lesions healed; 38 g. increase in wt.
802 ♂	Great wasting. Dermatitis of axillae and groins, spreading down inside of thighs and fore-limbs. Large raw area on abdomen	Egg-white 5 g.	3 weeks	Complete cure of all lesions; 31 g. increase in wt.
3075 ♀	Ophthalmia. Slight dermatitis of fore-paws. Cessation of growth	Egg-white 60 % of diet	3 weeks	Complete cure; 42 g. increase in wt.
757 ♀	Ophthalmia, very poor condition. Severe dermatitis of groins and axillae. Haematuria. Loss of weight	Egg-white extract (≡5 g. fresh egg-white)	4 weeks	Complete cure of all symptoms; 26 g. increase in wt.

growth experiments, Cheddar cheese and egg-white were tested. The former was not strongly curative as compared with milk; 2 g. daily of cheese (1·3 g. dry weight) was needed as compared with 6 cc. (0·7 g. dry weight) of milk. Egg-white was more potent; 5 g. (0·6 g. dry weight) produced a rapid amelioration of symptoms.

We have occasionally observed a very slow amelioration of symptoms taking place in rats kept throughout on the basal diet without any vitamin B_2 additions. In the above experiments, however, when improvement followed alterations in the diet it was striking and immediate and left little doubt of its cause.

Discussion of the relation of vitamin B_2 to the vitamin B complex, to the "P. P." (pellagra-preventive) factor, and to the factor preventing black-tongue in dogs.

It is by no means proved that vitamin B_2 is the only factor in the vitamin B complex other than that contained in Peters's antineuritic concentrate and several workers have brought forward evidence pointing to the existence of a third factor.

Hunt [1928] observed that an absorbable fraction (? vitamin B_1) and a filtrate (? vitamin B_2) from yeast, which could respectively supplement autoclaved yeast and whole wheat, could be further supplemented by whole fresh yeast or yeast residue minus the above fractions. We do not think, however, that in these experiments the possibility of a quantitative deficiency of either fraction was sufficiently excluded. The same criticism applies to Reader's observations [1928] that growth failed in rats whose vitamin B was supplied by Peters's antineuritic concentrate and large amounts of autoclaved marmite. Chick and Roscoe [1927] find that Peters's yeast concentrate in an amount equivalent to 0·6 g. yeast daily if supplemented by 0·4 to 0·6 g. autoclaved yeast supports normal growth.

We ourselves are more concerned with the possibility that in our experiments lack of growth and skin symptoms may not be due to the absence of the same factor. We can only say that those foods which promoted fair or good growth in young rats also protected from skin symptoms, and that in the curative experiments growth and healing ran parallel. The fact, however, that vitamin B_2 may be capable of further subdivision would not preclude its identity with Goldberger's pellagra-preventive "P. P." factor, itself possibly dual. In man two distinct conditions are described: pellagra, and *pellagra sine pellagra,* in which no skin eruption occurs. The "P. P." factor is effective against both.

In our experience the distribution of vitamin B_2 as determined by the method given above corresponds closely with that of the "P. P." factor and of the factor which prevents black-tongue in dogs, investigated by Goldberger, Voegtlin and others. The estimations made by these workers of the "P. P." factor contained in various foods are approximate, since in few cases was any attempt made to estimate the minimum necessary for protection, and the

basal diet was necessarily varied. Their results are collected in Table VI, the amounts given being expressed as the percentage of the dry weight of the diet, in order to obtain a rough comparison with our own results.

Table VI. *Comparison of the effects of various foodstuffs on vitamin B_2 deficiency in rats, on pellagra in man, and on black-tongue in dogs.*

Material	Rat Vitamin B_2	Man P.P. factor	Dog Black-tongue preventive factor
Maize endosperm	Very poor	Associated with pellagra in U.S.A. and Italy	—
Wheat flour	Very poor	Associated with pellagra in U.S.A.	Used in basal diet to produce the disease
Whole maize	Poor; 50 % less than normal growth	Traditionally associated with pellagra	No preventive action, used in basal diet to produce the disease
Whole wheat	Poor, but better than maize	—	66 % in diet delayed onset of the disease; no protection
Peas	30–40 % needed for good growth ("Clipper" peas)	30–35 % (168 g. daily); incomplete protection (Virginian cowpea)	58 %; slight protection, 73 %; almost complete protection (Virginian cowpea)
Wheat germ	15–30 % good growth	30 %; protection almost complete	31 %; protection almost complete
Fresh milk	5–10 % milk solids, good growth	20 % solids (1200 g. buttermilk daily) protected	250–300 cc. skim milk daily protected
Lean meat	7–10 % (dry wt.) good growth	10 % (dry wt., 50 g. daily) protected	11 % (dry wt.) protected
Dried yeast	1–3 % good growth	7 % (30 g. daily) protected	5 % protected
Liver	1–2 % (dry wt.) ox liver, good growth	Alcoholic extract of ox liver effective in treatment	11 % dried pork liver protected
Dried egg yolk	7–15 % good growth	—	17 % protected

Dried yeast is strongly preventive of pellagra; 30 g. daily averted the disease in asylum patients, otherwise liable [Goldberger and Tanner, 1925]. Fresh or autoclaved, it is also effective against black-tongue, if present in the proportion of 2–5 % of the basal diet [Goldberger *et al.*, 1928, 1].

Goldberger's original field studies in pellagra [Goldberger, Wheeler and Stucker, 1920] showed that "increasing supplies of milk or fresh meat were associated, one independently of the other, with a decreasing pellagra incidence," and later work has demonstrated the protective value of both foods against pellagra and against black-tongue [Goldberger *et al.*, 1924, 1926, 1928, 2]. Egg yolk, not yet tested in human pellagra, averts the canine disease [Goldberger *et al.*, 1928, 2]. Cowpeas gave some protection against both pellagra and black-tongue [Goldberger *et al.*, 1927, 1928, 2], but were not reckoned a good source of the protective factors.

An 85 % alcoholic extract of pork liver was used with good effect in the treatment of pellagra by Voegtlin [1920, Voegtlin *et al.*, 1920]. It was claimed

that a daily therapeutic dose equal to 1 kg. of fresh liver produced as good an effect as "a diet containing a considerable quantity of milk, eggs and meat." Dried pork liver is also preventive of black-tongue [Goldberger *et al.*, 1928, 2]. We found dried ox liver to be the most potent source of vitamin B_2 among the substances tested.

We found a mixed whole wheat, in which Manitoba wheat predominated, to have more vitamin B_2 potency than white or yellow maize. If vitamin B_2 and the "P. P." factor are identical, this result might explain the observed liability to pellagra of the maize eater rather than of the wheat eater, in localities where the whole grain and not highly milled products are consumed. Similarly, studies on black-tongue [Goldberger *et al.*, 1928, 1, 2] showed that the appearance of symptoms was earlier when the basal diet contained 78 % whole maize than when it contained 50 % whole wheat.

The endosperm of these two cereals, white flour and maize "grits," we found, on the other hand, to be about equally low in vitamin B_2 content. Therefore, if this vitamin be held to be a preventive of pellagra, white flour and maize endosperm should be equally conducive to the disease. This is possibly the case. Goldberger, Wheeler and Stucker [1920], in their field studies, found, when considering the poorest households with low supplies of fresh meat and milk, that no regular inverse ratio could be made out between the incidence of pellagra and the amount of white flour consumed. A rise in the household supply from 3 to 16 lbs. per adult male over a 15-day period did not cause any regular fall in the percentage of households affected by the disease. In the first report of the Thompson, McFadden Pellagra Commission [Siler, Garrison and MacNeal, 1913] it was stated that in the pellagra-affected districts studied, "wheat flour, in the form of bread or biscuit, is the principal bread-stuff, and corn-meal, while extensively used, is not nearly so staple an element of the dietary as wheat flour." It is possible that, where it is the custom to eat highly milled cereals, as in the United States, the more frequent association of pellagra with a maize than with a wheat diet is accidental, depending on other deficiencies in the diet of the maize eater. A maize diet is usually associated with poverty and marked by a shortage of such expensive foodstuffs as milk, meat and eggs.

Pellagra seems to be a disease of recent origin in the United States. According to Wood [1909], only sporadic cases were reported before 1909, and modern methods of milling are often held to be responsible for its recent increased prevalence. Our results support this to some extent, at any rate as far as wheat is concerned, since of all the cereal products examined we found wheat bran and wheat embryo richest in vitamin B_2.

Here again our results conform with those of Goldberger and his colleagues [1927, 1928, 2] on pellagra and black-tongue. A diet containing 60 % of maize germ meal cured two rats suffering from severe dermatitis, but since only poor growth occurred with a diet containing 30 %, maize germ meal must be considered a low source of the vitamin. Wood [1920] claimed to have fed

pellagrins on maize germ with prompt improvement. In our own experiments whole yellow maize as source of vitamin B_2 gave better growth than yellow maize "grits" (endosperm), showing that vitamin B_2 is lost in milling. This is more definitely the case with wheat, and it may be that the onset of pellagra in the United States coincided with the introduction of standard highly milled wheat and maize flour.

Evidence of the identity of vitamin B_2 deficiency in rats, of pellagra in man, and of black-tongue in dogs, is given in Table VI. The substances tested for the pellagra-preventive factor by Goldberger and his colleagues were given as daily additions to a daily diet of 400–500 g. (dry weight), equal to 2000–2500 calories. In the black-tongue experiments the test substances were incorporated in dietary mixtures of 400–500 g. (dry weight). From the tables given by these workers the percentages of the test substances in the diet have been roughly calculated.

There is very fair agreement in the effects of the various foods on the three conditions, and there seems little doubt that the condition produced in rats by a diet deficient in vitamin B_2 is the analogue of human pellagra. We suggest that our method might be useful in tackling any problems in the human disease that remain to be solved.

It is worth noticing that the rat, as compared with man and the dog, is relatively insusceptible to pellagra. Diets that produce florid pellagra in man and black-tongue in dogs do not cause dermatitis in rats, and only a rigorous purification of the basal diet can ensure the appearance of symptoms.

Summary.

(1) The method previously described by Chick and Roscoe [1928] has been used for estimating the vitamin B_2 value of foodstuffs. The special features of this method are (*a*) elaborate purification of the caseinogen in the basal diet, and (*b*) the use of Peters's antineuritic concentrate as source of vitamin B_1.

(2) The vitamin B_2 value of a substance was estimated by the minimum amount required to support normal increase in body-weight (11–14 g. per week) in young newly weaned rats receiving, apart from the test substance, a diet complete in all respects except vitamin B_2. For one week previous to the test the rats were maintained on a diet entirely free of both the B vitamins.

(3) Control rats from each litter used were fed on the basal diet, complete except for vitamin B_2. All remained stationary in weight and the majority developed skin symptoms.

(4) Some curative experiments on such rats suffering from dermatitis were carried out with materials containing vitamin B_2. In these amelioration of symptoms and resumption of growth were parallel.

(5) The vitamin B_2 value of wheat and maize is poor, that of maize being on the whole the lower in the samples examined. In wheat the germ and bran are better sources than the endosperm, and about equal to each other. In maize the germ is not so rich a source, but whole maize is better than maize endosperm.

(6) Dried peas have a low vitamin B_2 content.

(7) Dried ox liver, yeast and fresh whole milk are excellent sources of vitamin B_2. Dried meat and egg yolk are less good but richer than cereals.

(8) The relation of vitamin B_2 to Goldberger's "P. P." factor and to the factor preventing black-tongue in dogs is discussed. It is noted that so far the distribution of all three has been found to be identical.

(9) Some problems in the epidemiology of pellagra are discussed on the assumption that vitamin B_2 is identical with Goldberger's pellagra-preventive factor.

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LVI. A METHOD FOR THE ASSAY OF THE ANTINEURITIC VITAMIN B_1, IN WHICH THE GROWTH OF YOUNG RATS IS USED AS A CRITERION.

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THE antineuritic vitamin (vitamin B_1 in the nomenclature provisionally adopted by the Biochemical Society) was discovered in 1897 by Eijkman [1897] and was recognised by its ability to prevent and cure polyneuritis which developed in birds fed upon a restricted diet. The general distribution of this vitamin in natural foodstuffs was worked out by Eijkman and his colleague Grijns [1901] and quantitative assay was later attempted by Cooper [1913, 1914] and by Chick and Hume [1917,1, 2] by comparison of the minimal doses required to prevent (or cure) the polyneuritis of pigeons fed upon a diet of polished rice.

The antineuritic B_1 vitamin was formerly believed to be identical with, and is now known to be one constituent of, the complex "water-soluble B" discovered by McCollum and Davis [1915, 1] and found by them to be necessary in a diet to ensure normal growth in young rats.

It is now certain [Goldberger *et al.*, 1926; Chick and Roscoe, 1927] that the antineuritic vitamin B_1, equally with the second identified constituent of "water-soluble B," viz. the heat-stable, antidermatitis, (?) antipellagra, vitamin B_2, is necessary for normal growth of young mammals. Young rats just weaned are so sensitive to absence of vitamin B_1 that they seldom survive more than 3–4 weeks even if vitamin B_2 is provided, death usually taking place from inanition without symptoms of paralysis. If, however, the antineuritic vitamin is administered before the condition of the animal is too far advanced recovery takes place and normal growth is established. In rats the suddenness of death and the inconstancy of the symptoms following vitamin B_1 deficiency make it impossible to assay this vitamin by any method based upon cure or recovery in the acute stage. But if foodstuffs containing this vitamin are administered as soon as the animal's weight has become stationary the increase in body weight which follows can be used as a criterion for estimating the content of vitamin B_1 in the material fed.

The present method for assay of this vitamin has been developed along the lines of that previously described for the assay of vitamin B_2 [Chick and Roscoe, 1928] and used by Aykroyd and Roscoe [1929] in their study of the distribution of this vitamin. In that method the provision of vitamin B_1 unaccompanied by vitamin B_2 is conveniently arranged by administering

Peters's antineuritic concentrate prepared from yeast. But in the analogous method for assay of vitamin B_1 the provision of vitamin B_2 unaccompanied by the former is not so easy. We have used washed brewery yeast, autoclaved at 120° for 5 hours, which retains about one-half the vitamin B_2 potency of the original yeast and is at the same time practically devoid of vitamin B_1. Sometimes, however, traces of vitamin B_1 appear to survive this treatment, the result, doubtless, depending on the amount of vitamin B_1 contained in the original yeast. Another drawback to the use of autoclaved yeast is its tendency to cause diarrhoea. Further heating to destroy all traces of vitamin B_1 is not advisable, for the amount of the deleterious substances would thereby be increased, and as more vitamin B_2 would also be destroyed, larger doses would be required.

The occurrence of diarrhoea complicates experiments with B vitamins for two reasons. If severe, the animal naturally suffers apart from any dietetic deficiency; if slight, the loose stools passed do not fall cleanly through the meshed wire floor of the cage [Chick and Roscoe, 1928, p. 796] and more opportunity is afforded for faeces-eating, which is a common habit among rats deprived of B vitamins and tends to mitigate the effects of the deficiency [Steenbock, Sell and Nelson, 1923; Kennedy and Palmer, 1928].

A more convenient, but, for reasons stated below, less satisfactory, source of vitamin B_2 free from vitamin B_1 was found in the fresh white of hen's eggs, a surprising fact, since the yolk contains both factors in abundance. One can only conclude that the yolk membrane is impervious to the passage of vitamin B_1.

A basal diet (E.L. diet) was prepared, in which the purified caseinogen of our usual "-B" diet (P_2L diet) was replaced by egg-white (see Table I). The relative amounts of dry constituents were the same as in the P_2L diet, the protein amounting to 20 % in either case. In preparing the diet the egg-white is separated from fresh eggs and coagulated by heating in a double saucepan containing boiling water, put through a mincing machine and then thoroughly mixed with the dry constituents of the diet, the small extra amount of water being worked in afterwards. The prepared diet is steamed for 3 hours at 100° in order to cook the starch thoroughly and prevent the risk of "refection" [Fridericia *et al.* 1928; Roscoe, 1928].

Table I. *Basal diets used in the study of the (antineuritic) vitamin B_1.*

E.L. (egg-white) diet.	(g.)	P_2L (caseinogen) diet.	(g.)
Egg-white 800. Protein	100		
Water	700	Purified caseinogen	100
Rice starch	300	Rice starch	300
Cotton seed oil	75	Cotton seed oil	75
Salt mixture (McCollum's 185)	25	Salt mixture (McCollum's 185)	25
Distilled water	50	Distilled water	500
	1250		1000

0·4 g. autoclaved yeast daily was given to rats on diet P_2L to provide vitamin B_2. Cod-liver oil, to supply vitamins A and D, was administered to all rats separately by hand, 3 to 5 drops (0·05 g. to 0·1 g.) according to the size.

Young rats (40–50 g. weight), receiving the egg-white diet shortly after weaning, usually continued to grow for 2–3 weeks, after which the weight declined for lack of vitamin B_1. Death occurred if this was unchecked, but if Peters's antineuritic concentrate were given (0·1 cc. daily, equivalent to 0·6 g. dry yeast), the animals recovered and began to grow at the normal rate. In many cases they continued to grow normally for several weeks but a check was usually observed sooner or later before maturity was reached.

With young rats on the P_2L (caseinogen) diet supplemented with 0·4 g. autoclaved yeast daily to provide vitamin B_2, the preliminary period of growth generally lasted a week or two longer, owing possibly to traces of undestroyed vitamin B_1, which had survived the strong heating. In some

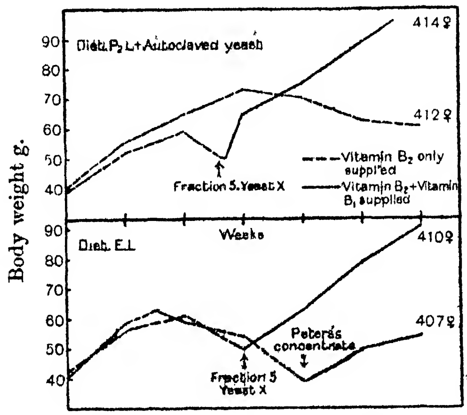

Fig. 1. Failure to grow of young rats from Litter 1184 on diets deprived of the antineuritic vitamin B_1, but containing egg-white (diet E.L.) and autoclaved yeast, respectively, as sources of vitamin B_2.
Rats 414 and 410 restored by equal doses of the same vitamin B_1-containing yeast extract; rat 407, by Peters's antineuritic concentrate.

instances the degree of growth displayed later during recovery, during the test of the vitamin B_1-containing material, was also greater than in rats receiving the egg-white diet. But in many cases concordant results were obtained when two rats of the same sex and litter, after suitable preparation on the two respective diets, received the same dose of vitamin B_1 (see Table II). This is illustrated by the weight curves of the four rats shown in Fig. 1. Two rats, 407 and 410, received E.L. diet and began to decline in weight after 2 weeks. After 4 weeks 407 was *in extremis* but was revived by a daily dose of Peters's antineuritic concentrate. Of the two rats on diet P_2L with a daily addition of 0·4 g. autoclaved yeast, 414 also grew for 2 weeks and then rapidly lost weight, but rat 412 was more resistant and attained the weight of 73 g. on this diet and after 6 weeks showed no sign of vitamin B_1 deficiency, although the body weight (68 g.) was then slowly falling.

Rats 414 and 410, after 2½ weeks and 3 weeks on their respective diets deprived of vitamin B_1, were treated with the same dose of a yeast fraction

containing this vitamin. The degree of growth induced was the same in both cases (see also Table II, Exp. 1).

In Table II are collected the results of a series of tests in which similar doses of vitamin B_1-containing materials were administered to pairs of rats prepared respectively on the two different basal diets. In series (*a*) the results are fairly concordant, in series (*b*) the rats receiving autoclaved yeast grew somewhat better than those on the egg-white diet.

It is hoped to make a survey of the antineuritic (vitamin B_1) content of some of the commoner foodstuffs for comparison (*a*) with that already made, using prevention and cure of polyneuritis of birds as a criterion, and (*b*) with the distribution of vitamin B_2 already published by Aykroyd and Roscoe [1929]. If the unit of vitamin B_1 be defined as the dose required to restore normal (11–14 g. weekly) growth to a young rat, of which the growth has failed on a basal diet deficient only in vitamin B_1 and containing excess of vitamin B_2, the assay of a foodstuff consists in determining the minimal dose necessary for this degree of restoration.

Table II. *Comparison of (antineuritic) vitamin B_1 assay using basal diets containing egg-white (diet E.L.) and autoclaved yeast (diet P.Y.) respectively as sources of vitamin B_2.*

Series	Exp.	Material	Daily dose	Litter	Rat	Body wt, g.	Diet	Weekly increase in wt, g.	Av.
(*a*)	1	Yeast Fraction X_5	Equiv. to 0·12 g. dry yeast	1184	410 ♀	50	E.L.	13, 16, 12	14
				,,	414 ♀	51	P.Y.	17, 12, 16	15
	2	,, XI_2 C	,, 0·25 ,,	1173	397 ♀	59	E.L.	9, 14	11·5
				1184	406 ♂	61	P.Y.	11, 14	12·5
	3	,, XII C	,, 0·25 ,,	1184	409 ♀	45	E.L.	21, 13, 14	16
				1173	396 ♀	39	P.Y.	16, 13, 7, 10	11·5
	4	,, XII C*a*	,, 1·0 ,,	1118	373 ♀	49	E.L.	22	22
				1117	361 ♂	43	P.Y.	23, 34	28
	5	,, XII_5	,, 0·12 ,,	1118	368 ♂	51	E.L.	20, 17, 11, 15, 14	15
				1117	360 ♂	40	P.Y.	18, 17, 7, 17, 11	14
(*b*)	6	,, XII_3 C	,, 0·25 ,,	1291	462 ♂	46	E.L.	15, 18, 20, 21	18·5
				1298	470 ♂	41	,,	17, 13, 18, 15	16
				1291	459 ♂	61	P.Y.	26, 21, 18, 13	19·5
				1298	468 ♂	54	,,	28, 10, 18, 15	18
	7	Dried egg yolk	0·5 g. = 1·9 fresh	1291	464 ♀	40	E.L.	2, 2, 1	2
				,,	461 ♀	51	P.Y.	7, 3, –1	3
		,,	1·0 g. = 3·9 fresh	1291	465 ♀	38	E.L.	16, 18, 13, 8	14
				1298	466 ♂	72	P.Y.	26, 19, 12, 9	18·5

The basal diet containing egg-white as source of vitamin B_2 would be in every way the more convenient of the two diets described, were it not that this diet, even when supplemented with adequate vitamin B_1, as Peters's antineuritic concentrate, does not maintain growth of rats to maturity. That egg-white contains in abundance the antidermatitis vitamin B_2 is shown by the many satisfactory cures of the "pellagrous" condition which have been obtained with comparatively small doses [Aykroyd and Roscoe, 1929]. It would seem that some third factor in the vitamin B complex, other than vitamins B_1 and B_2, is present in autoclaved yeast, and lacking in egg-white.

The heat stability of this hypothetical third substance is an argument against its identity with those suggested by the work of Williams and Waterman [1927, 1928] and of Reader [1928].

The matter is being investigated further. So far our observations do not permit a definite conclusion to be drawn upon the extent to which the absence of this hypothetical third factor may affect the validity of the test for vitamin B_1 described in this paper. In a few instances the growth of rats has become subnormal in the first few weeks after weaning, but it has usually remained unaffected until at least 6–10 weeks after weaning (about 9–13 weeks from birth), by which time the animal has attained a body weight of 100–130 g. No symptoms of ill health have been found in rats observed for three to four months after growth has become sub-normal. Nevertheless, pending the results of further investigation it seems safer, in the assay of vitamin B_1, to use autoclaved yeast as the source of vitamin B_2 in the diet rather than egg-white.

Summary.

1. Methods are described for assay of the antineuritic vitamin B_1, using growth of young rats as a criterion and basal diets in which the more heat-stable vitamin B_2 is supplied by fresh egg-white or by yeast autoclaved at 120° for 5 hours.

2. Fresh egg-white, while rich in vitamin B_2, is devoid of vitamin B_1. With autoclaved yeast there is a risk that traces of vitamin B_1 which have escaped destruction in the autoclave may be present. Nevertheless autoclaved yeast is to be preferred to egg-white for the reason given in 3, below.

3. Diets otherwise complete, which contain egg-white and Peters's antineuritic concentrate as sources of vitamins B_2 and B_1 respectively, are frequently found unable to maintain normal growth of rats beyond a few weeks after weaning. After this period growth is subnormal. It is suggested that a third hypothetical dietary factor may be present in the vitamin B complex, which factor is heat-stable, present in autoclaved yeast and lacking in egg-white.

4. If the unit of vitamin B_1 be defined as the amount which will restore normal growth (weekly increase in weight of about 10–14 g.) in young rats whose growth has failed on a basal diet deficient only in this vitamin, assay of a foodstuff for content of vitamin B_1 would consist in determination of the minimum dose necessary to restore this degree of growth.

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LVII. AN ATTEMPT TO SEPARATE VITAMIN B_2 FROM VITAMIN B_1 IN YEAST AND A COMPARISON OF ITS PROPERTIES WITH THOSE OF THE ANTINEURITIC VITAMIN B_1.

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YEAST contains both constituents of the "water-soluble B" vitamin, vitamins B_1 and B_2, in abundance, and seeing that the antineuritic (vitamin B_1) concentrate prepared by the method of Peters [1924] is free from vitamin B_2 [Chick and Roscoe, 1927] our first endeavour towards the separation of this vitamin was to scrutinise every stage of the process to find where the disappearance occurred.

Our method has been that of Kinnersley and Peters [1925, 1927] with a few modifications. 10–12 kg. of pressed, brewery yeast were washed three or four times with cold tap water. The pressed yeast was puddled with water to the consistency of cream, filtered on a Gooch filter, using slight suction, and finally sucked dry or pressed in a hand press, this process being repeated three or four times until the filtrate was of a pale straw colour. After removal of a small sample for determination of the dry weight, the washed, pressed yeast (in the proportion of 1 kg. pressed, or 200 g. dry, yeast to 2–3 litres water) was thrown into boiling tap water containing 0·02 % acetic acid; this was raised again to the boiling point and allowed to boil for 5 minutes and filtered. This extract ("Fraction 5") about 20–30 litres in volume, was then treated with 25 % lead acetate solution till no more precipitate was formed (*ca.* 55–80 cc. per litre of extract), the whole allowed to stand overnight and then filtered. To the filtrate ("Fraction 4") baryta, at first solid and then as saturated solution, was added, till no more precipitate formed. As the resulting solution is alkaline, filtration was carried out at once and the filtrate acidified with strong H_2SO_4 until a p_H of about 4·0 was reached (using the indicator bromocresol green or Congo red). The solution, heated to assist agglutination of the precipitated $BaSO_4$, was filtered, and to the filtrate Hopkins's reagent (10 % solution of $HgSO_4$ in water containing 70 cc. H_2SO_4 per litre) was added to the point of most complete precipitation (25–50 cc. per litre of filtrate). In order to remove any traces of lead and mercury that might be present, H_2S was passed through the filtrate to saturation and the whole allowed to stand several hours in corked bottles; on heating the solution,

the metallic sulphides settled well and could readily be filtered. If the previous precipitation with baryta is omitted, these sulphides tend to remain in the colloidal form and are separated with difficulty. The reaction of the filtrate was then carefully adjusted to the neutral point (using litmus or phenol red) and treated twice with norite charcoal which had been purified before use by boiling with dilute hydrochloric acid and thorough washing with distilled water to remove all traces of acid. The charcoal was filtered off, washed and sucked dry on a Gooch filter and the filtrate treated with a second quantity. 60–70 g. norite per kg. dry yeast were used in each adsorption. Both portions of charcoal were boiled two or three times with fresh quantities of dilute acid alcohol (50 cc. alcohol, 50 cc. water, 1 cc. concentrated hydrochloric acid) and the combined extracts reduced to a convenient bulk (the equivalent of 6 g. yeast contained in 1 cc.) by distilling under reduced pressure at a temperature of 40 to 50°.

After each precipitation a portion of the filtrate was set aside and examined by the method already published [Chick and Roscoe, 1928] for its content of vitamin B_2. It is necessary to work quantitatively, determining in each case the minimum dose required to maintain a standard degree of growth (a weekly increase in body weight of 11–14 g.) and referring the dose given to the equivalent amount of the original dried yeast.

Table I. *Details of preparation from Yeast V of Peters's antineuritic vitamin B_1 concentrate, and results of tests of filtrates at each stage for content of vitamin B_2*.*

Process	Material tested	Dose given, expressed as equivalent of dry yeast g.	No. of rats observed	Average growth g. per week
	Dried yeast (from Table III, Chick and Roscoe [1927])	0·2	—	11
		0·4	—	23
1. Extraction of pressed, washed Yeast V with boiling water containing 0·01 % acetic acid	*Fraction* 5. Filtrate from 1 after evaporation to small bulk	0·25	3	12
		0·5	3	21
		1·0	3	29
2. Precipitation with Pb acetate at p_H 4·7 (800 cc. 25 % Pb acetate soln. per kg. dry yeast)	*Fraction* 4. Filtrate from 2 after evaporation to small bulk and removal of Pb with H_2SO_4	1·0	2	14
3. Precipitation with baryta and acidification with H_2SO_4 to p_H *ca.* 4·0 to precipitate $BaSO_4$	*Fraction* 3. Filtrate from 3 after evaporation and partial neutralisation with removal of any further traces of $BaSO_4$	2·0	2	6
		3·4	2	15†
4. Precipitation with acid mercuric sulphate, Hopkins's reagent (260 cc. per kg. dry yeast), and removal of traces of Hg by H_2S	*Fraction* 2. Filtrate from 4 after evaporation to small bulk	1·0	1	0
		2·0	1	0
		3·0	1	2
5. Adsorption of vitamin B_1 by treatment with norite (100 g. norite per kg. dry yeast, in two portions) after neutralisation	*Fraction* 1. Filtrate from norite after evaporation to small bulk	4·0	2	2·5

* Method of Chick and Roscoe [1928]. Growth of young rats observed for 2–4 weeks on a diet deprived of B_2 vitamins, but receiving vitamin B_1 as 0·1 cc. (=0·6 g. yeast) Peters's antineuritic concentrate.

† A similar fraction prepared from yeast VII was used for part of this test. These tests followed on those of the 2·0 g doses.

In Table I are set out the details of one such preparation, viz. from Yeast V. There was little loss of vitamin B_2 in Fraction 5, the first extract

from the yeast with acidified boiling water. The average weekly growth-increments of young rats receiving daily doses equivalent to 0·25 g. dried yeast were 12 g., about the same as that of rats receiving 0·2 g. daily of dried yeast; daily doses equivalent to 0·5 g. dried yeast had about the same effect as 0·4 g. dried yeast.

During precipitation with lead acetate there was a loss. Daily doses of the filtrate, Fraction 4, equivalent to 1·0 g. dry yeast produced an average weekly increase in weight of only 14 g. in two male rats, whereas an equivalent amount of Fraction 5 induced about twice as much growth (29 g.).

The precipitation with baryta caused a further loss in vitamin B_2 and, after precipitation with mercuric sulphate and subsequent removal of traces of mercury from the filtrate with SH_2, the filtrate contained no significant amount of this vitamin. Doses equivalent to 2 or 3 g. of the original dried yeast did not produce more growth than occurred in some control animals on the basal diet.

One may, therefore, conclude that about one half to three-fourths of the vitamin B_2 contained in the original yeast is carried down with the lead acetate precipitate; of the remainder, the greater part (about two-thirds) disappears with the precipitate formed by baryta, and the rest with that given with acid mercuric sulphate. Attention was, therefore, directed to the precipitation with lead acetate.

Influence of hydrogen ion concentration on the amount of vitamin B_2 carried down by lead acetate.

In the Peters process as described above, the precipitation takes place at a p_H of about 4·5–4·7 (bromocresol green). We tried precipitation at p_H 2·6 (bromophenol blue), this being the degree of acidity attained during the precipitation with Hopkins's reagent in the above process, when the last remaining traces of vitamin B_2 were found to be adsorbed. Precipitation with lead acetate at p_H 6·3–8·9 (using bromocresol purple and phenol red as indicators) was also tried.

At p_H 2·6 less than one-half the vitamin B_2 present was removed by lead acetate, at p_H 4·7 about three-fourths. In neutral or slightly alkaline solution the experiments indicated that all was carried down. Whereas of the original extracts the equivalent of 0·25 g. to 0·5 g. dry yeast, respectively, supported normal growth, little or no growth was induced by doses of the filtrates equivalent to 1·0 or 2·0 g. dry yeast (for details see Table II, Fractions 4, Yeast VIII and Yeast X). Unfortunately, as will emerge later, none of these precipitates with lead acetate was consistently free from vitamin B_1.

Recovery of vitamin B_2 from the lead acetate precipitate.

Vitamin B_2 can be recovered by decomposing the lead acetate precipitate with sulphuretted hydrogen. If the reaction is acid, however, the lead sulphide remains in colloidal solution, owing to the action of the yeast gum which

possesses an alkaline isoelectric point. If the solution is brought to p_H 8–9 (thymol blue) the lead sulphide separates readily, but in that case vitamin B_2 is adsorbed on the precipitate and the solution correspondingly impoverished. To prevent loss in this way the reaction should not be less acid than p_H 3·0 (bromophenol blue). The technical trouble caused by the yeast gum at a higher p_H can be overcome by hydrolysing it in the original extract.

Table II. *Influence of hydrogen ion concentration upon the removal of vitamin B_2 (and B_1) from an extract of brewers' yeast by precipitation with lead acetate.*

Estimation of vitamins B_2 and B_1 in the filtrates and in the solutions obtained by decomposition of the precipitate by methods of Chick and Roscoe [1928] and [1929] respectively.

Material	Dose given expressed as equivalent of dry yeast (g.)	Vitamin B_2 content: No. of rats observed	Vitamin B_2 content: Av. growth g. per week	Vitamin B_1 content: No. of rats observed	Vitamin B_1 content: Av. growth g. per week	Dose (expressed as equivalent in g. of dry yeast) required to give normal (11–14 g. weekly) increase in weight as source of Vitamin B_2	as source of Vitamin B_1
Yeast VIII:							
Fraction 5. Extract with dilute acetic acid	0·12	2	7	—	—	0·25	—
	0·25	4	11	—	—		—
	0·5	1	18	—	—		—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 2·6	0·5	3	13	—	—	0·5	—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 4·7	1·0	1	13	—	—	1·0	—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 6·3	0·5	1	0	—	—	No growth with 1·0	—
	1·0	1	0	—	—		—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 8·9	1·0	3	0	—	—	,,	—
Yeast X:							
Fraction 5	0·25	4	9	—	—	0·25 to 0·5	—
Fraction 5, after hydrolysis 1 hour 100° at p_H 1·5	0·5	3	13	—	—		
	0·25	1	13	—	—		
Fraction 4. Filtrate after precipitation with Pb acetate at p_H 7·2	1·0	1	7	—	—	>2·0	—
	2·0	3	4	—	—		
Fraction C_1 by decomposition with SH_2 of precipitate formed with Pb acetate at p_H 4·7	0·5	1	10	1	1	0·5 to 1·0	>2·0
	1·0	1	11	1	0·2		
	2·0	—	—	1	0·3		
Fraction C_2 by decomposition with SH_2 of precipitate formed with Pb acetate at p_H 7·5	0·5	3	11	1	7·5	0·5 to 1·0	0·5 to 1·0
	1·0	1	14	1	14		
Yeast XI:							
Fraction 5	0·25	2	11	—	—	0·25	—
Fraction C_1, from Pb acetate precipitate formed at p_H 4·7	0·25	2	11	2	12	0·25	0·25
	0·5	2	9	1	18		
	1·0	1	19	—	—		
Fraction C_2, from Pb acetate precipitate formed at p_H 7·5	0·5	2	9	—	—	0·5 to 1·0	<1·0
	1·0	1	15	1	25		
Yeast XII:							
Fraction 5	0·12	—	—	2	15	0·25 to 0·4	0·12
	0·25	2	8·5	—	—		
	0·4	2	14	—	—		
Fraction C_2, from Pb acetate precipitate formed at p_H 7·3	0·25	—	—	4	15	0·5	0·25
	0·5	6	14·5	1	20		

The following procedure was adopted. To 1 litre of the original dilute acetic acid yeast extract (Fraction 5) were added 10 cc. concentrated hydrochloric acid, making the p_H about 1·5 (thymol blue). The mixture was heated in steam at 100° for 1 hour, neutralised, the p_H adjusted to about

7·5 (phenol red) and 10 % solution of basic lead acetate added till precipitation was complete. The liquor was decanted, the precipitate drained on a Gooch filter and washed with a small amount of distilled water. If not wanted immediately the precipitate can be dried and worked up later, but if kept in the moist condition it is liable to become infected with moulds. The precipitate was suspended in water, the reaction adjusted to p_H 3·0 (bromophenol blue), sulphuretted hydrogen passed to saturation and the whole shaken for about 2 hours. After standing, a clear yellow fluid should separate from the lead sulphide. This was filtered, the residue washed, again suspended in water and treated with sulphuretted hydrogen, the operation being repeated two or three times until the top fluid was colourless. The combined filtrates and washings, which were strongly acid, were neutralised with sodium hydroxide or baryta (preferably the latter) to *ca.* p_H 3·0 and evaporated on a water-bath to a convenient bulk (1–2 cc. containing the equivalent of 1 g. yeast), during which a small additional amount of lead sulphide was often deposited. This degree of heating does not seem to impair the activity of the preparation. The sodium sulphate present in this final product, referred to as Fraction C for brevity, sometimes caused diarrhoea in the doses administered to the rats; it can, however, be replaced by sodium chloride if precipitated by barium chloride, but care must be taken to avoid any excess.

Table II gives the details of such preparations made from Yeasts X, XI and XII respectively. In addition to the tests for vitamin B_2, the products, Fractions C, were also tested for content of vitamin B_2, using the method described in the accompanying paper [Chick and Roscoe, 1929].

The Fractions C_2, obtained from Yeasts X and XI by decomposition of the lead acetate precipitates formed at p_H 7·3 to 7·5, contained about one-half of the vitamin B_2 present in the respective original yeast extracts (Fractions 5). Fraction C_2, obtained in the same manner from Yeast XII, contained nearly the whole of the vitamin B_2 of the corresponding Fraction 5; normal growth was obtained with the equivalent of 0·5 g. yeast, the dry weight of the dose (less ash) being 0·03 g. It was, however, admixed with vitamin B_1 and although it contained only about one-half of that present in the original Fraction 5, it was richer in this vitamin than in vitamin B_2.

By precipitation with lead acetate in neutral or alkaline solution more vitamin B_1 is carried down than in the weakly acid solution (p_H 4·7) of Peters's original process. It was hoped that by precipitation at p_H 4·7 a method of separation would be obtained. From Yeast X a Fraction C_1, almost free from vitamin B_1 was indeed obtained from the lead acetate precipitate formed at this p_H. However, this was not found to occur uniformly (compare Fraction C_1 prepared from Yeast X with Fraction C_1 from Yeast XI, Table II). The reason for the discrepancy is not obvious but may be due to differences in the relative proportions of the two vitamins contained in the original yeast extract.

It is possible that by working throughout in more acid solutions, at p_H 2·0

to 3·0, a preparation of vitamin B_2 free from B_1 might be obtained, but it would be very weak. In the experiments made with Yeast VIII (Table II), when the lead acetate precipitation occurred at a low p_H, the amount of vitamin B_2 adsorbed was much reduced.

Yeast extract seems to be an unfavourable medium for separation of these two vitamins by the type of process here employed, and it is possible that success might be attained by working with some other material. Rosedale [1927] used a method resembling Peters's for preparing an antineuritic concentrate from rice polishings. On decomposing the lead acetate precipitate with sulphuretted hydrogen, he obtained a substance which maintained health and weight in pigeons but had no curative effect on polyneuritis, although affording temporary relief by causing evacuation of the bowel. At the present time little is known, except by inference, of the relation of vitamin B_2 to the nutrition of the pigeon, but it is probable that Rosedale's preparation contained vitamin B_2 without admixture of vitamin B_1.

Other methods tried for separating vitamin B_2 from B_1 in yeast extracts.

The vitamin B_2 preparations described above could doubtless be freed from vitamin B_1 by heating at a high temperature, but the vitamin B_2 content would be reduced at the same time. The vitamin B_1 in yeast was destroyed and the vitamin B_2 reduced to about one-half the original amount by heating to 120° for 5 hours [Chick and Roscoe, 1927, Table III].

Our endeavour was to effect a separation by other means than heat, and to this end we tried the following: (1) dialysis, (2) solubility in strong alcohol and (3) ultra-violet light. None was successful, but as the work afforded some information as to the properties of these two vitamins it seems worth while to place it on record.

Dialysis.

Fraction 5 (acetic acid extract) from Yeast X, which contained both vitamins B_1 and B_2, was placed in a bag of cellophane (a cellulose membrane prepared from viscose) and dialysed against distilled water. The solutions were acidified to p_H about 3·0) and dialysis was carried on in a refrigerator to prevent putrefaction. After 4 days, the inside and outside liquors were tested for content of vitamins B_1 and B_2 respectively. No difference could be detected in the concentration of vitamin B_2 in the fluid inside and outside the membrane. Vitamin B_1 also passed freely through the membrane (see Table III, exp. 1).

Solubility in alcohol.

There are many facts in the literature indicating that the antineuritic (vitamin B_1) component of the complex "water-soluble B" is more readily soluble in strong alcohol than the more heat-stable (vitamin B_2) component.

Table III. *Influence of dialysis, ultra-violet irradiation, and alcohol upon vitamins B_1 and B_2 respectively contained in a dilute acetic extract* (*Fraction* 5) *from yeast.*

Methods of assay as in Tables I and II.

Exp.	Material	Dose given expressed as equivalent of dry yeast (g.)	Vitamin B_2 content: No. of rats observed	Vitamin B_2 content: Average growth g. per week	Vitamin B_1 content: No. of rats observed	Vitamin B_1 content: Average growth g. per week
1	*Dialysis:*					
	Yeast X, Fraction 5 (extract in dilute acetic acid)	0·25	4	9	—	—
		0·5	3	13	—	—
	Yeast X, Fraction 5. Dialysed: outside liquor	0·25	1	8	1	10
		0·5	1	12	1	14
	inside liquor	0·25	1	8	—	—
		0·5	1	15	—	—
2	*Effect of ultra-violet light:*					
	Yeast XII, Fraction 5	0·12	—	—	2	15
		0·25	2	9	—	—
		0·4	4	14	—	—
	Yeast XII, Fraction 5. Irradiated with ultra-violet light for 6 hours	0·12	—	—	2	11·5
		0·25	1	4	—	—
		0·5	1	8	—	—
	for 12 hours	0·12	—	—	2	7
		0·25	—	—	1	8
		0·5	1	4	—	—
		0·75	1	9	—	—
	Solubility in alcohol:					
	Yeast XII, Fraction 5. See above exp. 2.					
	Yeast XII, Fraction 5, after drying and exposure to air for 19 days	0·4	2	13·5	—	—
	Yeast XII, Fraction 5, after drying and exposure to 94 % alcohol for 19 days	0·4	2	11	—	—
	Yeast XII, Fraction 5. Fraction soluble in 92 % alcohol	0·25	—	—	2	12
		0·5	2	2	—	—
		1·0	2	0·5	—	—
	Yeast XII, Fraction 5. Fraction insoluble in 92 % alcohol	0·12	—	—	1	10
		0·25	—	—	1	16
		0·5	3	9	—	—

The antiberiberi, antineuritic vitamin, has long been known to be soluble in alcohol in strengths ranging from 88 % to absolute [Fraser and Stanton, 1910; Eijkman, 1911; Chamberlain and Vedder, 1911; Kinnersley and Peters, 1925, and others]. It was further observed by Schaumann [1911] that extracts made with 96 % alcohol from yeast or rice bran, while potent in the cure and prevention of polyneuritis, could not maintain the body weight of the experimental animals, whereas equivalent amounts of the original materials could do both. Drummond [1917] found it impossible to obtain a satisfactory extract of the complex formerly known as "water-soluble B" with absolute alcohol, but succeeded with 70 %. This discrepancy in alcohol-solubility between the antineuritic vitamin and the complex water-soluble B was one of the facts throwing doubt on the supposed identity of these two dietary factors [Mitchell, 1919].

After demonstrating the existence of the two vitamins in water-soluble B, Goldberger and his colleagues [1926] found an 85 % (by volume) alcoholic extract of white maize to be rich in the antineuritic vitamin B_1, but deficient

in the heat-stable P-P (vitamin B_2) component. They write, "we have gained the impression that 'P-P' is relatively much more soluble in acidulated water than in 85 % alcohol, whereas the antineuritic factor is soluble in both." In a recent paper Sherman and Sandels [1929] find vitamin B_2 ('vitamin G') to be insoluble in 95 % alcohol.

We have fully confirmed the conclusions of Goldberger and his colleagues and consistently find vitamin B_1 soluble, and vitamin B_2 insoluble, in alcohol of concentrations from 83 to 93 % by weight. But we have not been able to effect a satisfactory separation of the two vitamins in this manner. After the final recovery from the insoluble residue the vitamin B_2 was reduced in amount, suggesting that this vitamin was destroyed by contact with alcohol or by some other conditions experienced during the operation. Sherman and Sandels [1929], whose work appeared after these experiments were complete, came to a like conclusion.

The following illustrates the method used. Fraction 5, the dilute acetic acid extract from Yeast XII, was concentrated on a water-bath. Of the concentrate, 50 cc., equivalent to 82 g. yeast, were dropped slowly into 2½ litres of 94 % (by weight) alcohol with vigorous stirring. A precipitate was formed immediately. The mixture was shaken for 2 hours and allowed to stand several days after which the clear yellow top liquor was siphoned off. The insoluble matter was filtered and, after washing, shaken with a fresh amount of 94 % alcohol and the operation repeated until the alcoholic extract was colourless.

The combined alcoholic extracts and washings, of which the specific gravity corresponded to strengths of alcohol varying from 91·5 to 93 % alcohol, were concentrated to a small bulk at a low temperature under reduced pressure and finally taken to dryness at 37° with a fan. This dry residue, which was very acid, and the dried insoluble precipitate (= 7·7 g.) were dissolved in water and separately tested for content of vitamins B_1 and B_2 respectively.

The results (Table III, exp. 3) showed that about one-half the vitamin B_1 present in the original material was in the alcohol-soluble fraction. Vitamin B_2 was not detected in this fraction, but the amount present in the alcohol-insoluble portion was only equal to about one-half of that originally present.

In order to see whether this destruction of vitamin B_2 were due to the contact with alcohol or to oxidation when in the dry condition, a sample of the concentrated yeast extract used for the above experiment was evaporated to complete dryness and portions were allowed to remain at room temperature exposed to the air, and covered with 94 % alcohol respectively. After 19 days the alcohol was blown off the latter at 37° with a fan and the dry residue taken up in water and tested for vitamin B_2 content. This was compared with that of both the original solution and the solution made from the air-dried material. The results, set out in Table III, exp. 3, showed that a slight diminution in potency had taken place in the material which had been in contact with alcohol, but the difference was not significant and seemed too small to account for the loss experienced in the experiment described above.

Further investigation is needed to elucidate this matter. It may be that the destructive effect of alcohol on vitamin B_2 depends on the reaction of the solution.

Action of ultra-violet light.

Hogan and Hunter [1928] reported experiments on growing rats in which "vitamin B" was supplied in the diet respectively by autoclaved yeast or yeast irradiated by ultra-violet light. From the results they concluded that the heat-labile antineuritic (vitamin B_1) component was resistant to the action of ultra-violet light, whereas the heat-stable component (vitamin B_2) was destroyed by irradiation. If this were so, an easy method would be available for preparing an antineuritic material free from vitamin B_2, which could replace Peters's concentrate in the study of vitamin B_2.

The action of ultra-violet light was tested upon the dilute acetic acid extract, Fraction 5, from Yeast XII, which is rich in both vitamins (Table III). The material was concentrated to a small volume (2 cc. containing the equivalent of 1 g. yeast) and poured over the surface of a flat white dish, to an average depth of about 2 mm. and irradiated at a distance of 40 cm. from a mercury vapour arc lamp. From time to time a little distilled water was added to prevent drying and the whole well mixed. After 6 hours' irradiation the content of vitamin B_2 was found to be reduced to about one-half, while that of vitamin B_1 had also suffered, though to a less extent. After 12 hours' irradiation, more than half the vitamin B_1 originally present had been destroyed. Further destruction of vitamin B_2 had also occurred, but this was by no means complete (see Table III, exp. 2).

Summary.

1. The Peters process, by which an antineuritic, vitamin B_1, concentrate free from vitamin B_2 is prepared from yeast, has been scrutinised to find out at which stage the removal of vitamin B_2 occurs.

2. About one-half to three-quarters of the vitamin B_2 present in the original yeast was carried down in the precipitation with lead acetate at p_H 4·7. The removal was more complete if the precipitation were carried out in neutral or slightly alkaline solution.

3. If the lead acetate precipitate is decomposed with sulphuretted hydrogen, a clear solution containing vitamin B_2 can be separated from the lead sulphide if certain precautions be taken.

The dose of this preparation required to maintain normal growth in young rats on diets deprived only of vitamin B_2, contained 0·03 g. dry weight (less ash) and was the equivalent of 0·5 g. of the original yeast.

4. This preparation contained more or less vitamin B_1, depending on the reaction at which precipitation with lead acetate had been carried out and probably on the amount (relative to vitamin B_2) contained in the original yeast.

5. Vitamin B_2 is insoluble, and vitamin B_1 is soluble, in alcohol of 92 % by weight. The method employed to effect a separation with strong alcohol destroyed vitamin B_2. The attempt to prepare a vitamin B_2 concentrate free from vitamin B_1, by the use of alcohol, was unsuccessful.

6. Both vitamins dialyse freely through cellophane (a viscose preparation of cellulose).

7. Ultra-violet light was found to exert a destructive action on both vitamins, vitamin B_2 being destroyed at a quicker rate than vitamin B_1. The result obtained with vitamin B_2 confirms the previous observation of Hogan and Hunter [1928]. Their conclusion that vitamin B_1 is resistant to ultra-violet light is not, however, confirmed.

In conclusion we desire to express our thanks to Sir Charles Martin for helping us by his continued advice and criticism.

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LVIII. THE EFFECT ON VITAMIN B_2 OF TREATMENT WITH NITROUS ACID.

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IN 1918 McCollum and Simmonds reported that the dietary factor "water-soluble B" was resistant to the action of nitrous acid, the activity of the vitamin being tested by its power to restore growth in rats where increase in weight had failed on a diet deficient in this vitamin [1918]. Levene and Van der Hoeven [1926] confirmed this result. They found that the Osborne-Wakeman fraction prepared from yeast contained 90–100 % of its original "vitamin B" potency after deamination. Their tests also involved observations of the growth of rats [Levene and Van der Hoeven, 1924], but as the authors themselves admit [Levene, 1928], the time during which the rats were under observation (3–4 days) was too short for trustworthy results. McCollum and Simmonds observed the growth of their experimental animals over a period of 2 weeks.

Although the above experiments were made before the composite nature of the "water-soluble vitamin B" was recognised, it would be natural to infer that treatment with nitrous acid had not destroyed either of the two known components, vitamins B_1 and B_2, since both are necessary for growth. Peters [1924] found his antineuritic, vitamin B_1, concentrate prepared from yeast to be completely resistant to the action of nitrous acid as tested by its capacity, before and after treatment respectively, to cure polyneuritis in pigeons, but in a recent paper, Levene [1928] reports the opposite to be true of the heat-stable, antidermatitis, vitamin B_2. A vitamin B_1 concentrate, prepared by adsorption with silica gel from the Osborne-Wakeman fraction of yeast, was found to contain a small proportion of the heat-stable, vitamin B_2, factor, which could be removed by deamination. The activity of this vitamin is stated to be destroyed by treatment with nitrous acid, while the heat-labile (vitamin B_1 factor) remains intact. No description is given of the animal tests on which this statement is based.

The suggestion that the activity of vitamin B_2 might be dependent upon the presence of amino-nitrogen seemed important enough to be worth confirmation. Accordingly, the action of nitrous acid was tested on a vitamin B_2 concentrate prepared from yeast by decomposition with sulphuretted hydrogen of the precipitate formed when lead acetate is added to a dilute

acetic extract of washed brewery yeast [Chick and Roscoe, 1929]. This concentrate contained about 6 % of the dry weight, 3 % of the nitrogen and about one-half the vitamin B_2 of the original yeast. The daily dose required to maintain normal growth (weekly increase in weight of 11–14 g.) in young rats on a diet deprived only of vitamin B_2 was 0·03 g. (dry weight, less ash), and was contained in the equivalent of 0·5 g. of the original yeast. Vitamin B_1 was also present.

Deamination was carried out as follows, using the method described by Peters [1924]. To 50 cc. of the preparation (equivalent to 50 g. original dried yeast), which was already acid (p_H *ca.* 3·0), 50 cc. distilled water, 5 cc. of a 30 % solution of sodium nitrite and 10 cc. of 10 % sulphuric acid were added. The mixture was shaken by hand for 5 minutes, left to stand overnight, heated next day on a water-bath, and after all foaming had ceased, allowed to boil for 10 minutes. Estimations by Van Slyke's method (15 mins. shaking) gave 0·091 % amino-nitrogen (*i.e.* 0·091 g. per 100 g. of the original yeast) in the material before treatment, and this was reduced to about one-sixth, viz. to 0·016 % amino-nitrogen after treatment.

Table I. *Influence of deamination upon the vitamin B_2 activity of a preparation (XII_3C) obtained from brewer's yeast, daily dose* 0·5 *cc., equivalent to* 0·5 *g. dried yeast.*

The rats received diet P_8L deprived of B vitamins supplemented by daily doses of cod-liver oil (0·05–0·1 g.), to provide vitamins A and D, and of Peters's antineuritic concentrate (0·1 cc. equivalent of 0·6 g. yeast), to provide vitamin B_1.

Material	Nitrogen in preparation (reckoned per 100 g. of original yeast) Total	Amino	Litter No.	Rat No.	Body wt g.	Increase in body weight in successive weeks g.	Average	Mean
Before treatment with nitrous acid	0·24	0·091	1264	451 ♀	36	15, 12, 13	13·3	14·0
			1285	452 ♂	41	16, 11, 15	14	
			,,	455 ♀	35	18, 14, 12	14·7	
After treatment with nitrous acid	0·13	0·016	1264	450 ♀	40	15, 14, 12	13·7	13·8
			1285	453 ♂	43	16, 17, 11	14·7	
			,,	454 ♀	37	12, 13, 14	13	

No diminution in the vitamin B_2 potency of the preparation could be detected. The results of the tests, using our method of vitamin B_2 assay [Chick and Roscoe, 1928], are set out in the accompanying Table I. Six young rats (35–43 g. weight) were divided into two groups, similar in respect of litter and sex, and maintained on a basal diet deprived of B vitamins with the addition of a daily dose (0·1 cc., equivalent to 0·6 g. yeast) of Peters's antineuritic concentrate, to provide vitamin B_1. One group of rats received the vitamin B_2 concentrate before treatment with nitrous acid (daily dose equivalent to 0·5 g. original dried yeast) and the second an equal dose of the material after deamination. Growth of both groups was observed for 3 weeks. The rats in the first group showed an average weekly increase in weight of 14·0 g. and those in the second of 13·8 g., proving that no destruction of vitamin B_2 had taken place during treatment with nitrous acid.

SUMMARY.

Levene's observation that the activity of vitamin B_2 is destroyed by the action of nitrous acid is not confirmed. A vitamin B_2 preparation from yeast was found to possess equal power after and before treatment to induce growth in young rats on a diet deprived of this vitamin.

My thanks are due to Miss M. H. Roscoe for her care of the experimental animals used in the tests.

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LIX. THE APPLICATION OF THE IODIMETRIC METHOD TO THE ESTIMATION OF SMALL AMOUNTS OF ALDOSES.

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(*Received May 7th, 1929.*)

One of the most interesting facts emerging from the study of the carbohydrate-phosphoric esters formed during alcoholic fermentation is the rapid conversion of glucose into derivatives of fructose and *vice versa*. The fermentation of either glucose or fructose by yeast juice, dried yeast or zymin may give rise to phosphoric esters of glucose, γ-fructose, trehalose and possibly of other sugars, in proportions which vary within wide limits according to the type of yeast and other conditions of the fermentation. For the investigation of these products a reliable method for distinguishing quantitatively between aldoses and ketoses is much to be desired.

The reaction between aldoses and alkaline solutions of iodine

$$R.CHO + I_2 + 3NaOH = R.COONa + 2NaI + 2H_2O$$

was first applied to the estimation of sugars by Romijn [1897] who, after comparative experiments, selected borax as the most suitable form of alkali. At 25° oxidation of glucose and other aldoses was found to be complete in 16–22 hours, but ketoses and non-reducing sugars were also oxidised to a varying extent. Bland and Lloyd [1914] employed a solution containing iodine and sodium hydroxide in equimolecular proportions, and claimed that, at room temperature, oxidation of glucose, maltose and lactose was complete in 5 minutes, while fructose and sucrose were not attacked. Bougault [1917] and Cajori [1922] used sodium carbonate as the alkali and allowed the reaction to proceed for 20–30 minutes at room temperature. Both emphasise the necessity of employing a large excess of iodine (three times the theoretical quantity). Colin and Liévin [1918] replaced the carbonate by disodium hydrogen phosphate without any obvious advantage. In Willstätter and Schudel's [1918] modification sodium hydroxide is used and is added last, in the proportion of 3 mols. to 2 mols. of iodine—as required by the equation. The oxidation is carried out at room temperature during 12–20 minutes. This modification has been most frequently employed in biochemical investigations but the authors' claim that under the specified conditions fructose and sucrose are not attacked has been disputed by Judd [1920] and Bruhns [1923], who have obtained values for fructose varying between 7 % and 11 %

in terms of the reducing power of glucose. Baker and Hulton [1920] found that oxidation of aldoses was complete in 3–5 minutes, but even under these conditions fructose was oxidised to the extent of 7 %. According to Judd this partial oxidation is the result of the Lobry de Bruyn and van Ekenstein transformation but the experimental evidence brought forward by Baker and Hulton does not bear out this suggestion.

The investigations of Kolthoff [1923] and of Hinton and Macara [1924] show that the degree of oxidation both of aldoses and of ketoses is influenced to a marked extent by the relative proportions of iodine and alkali, and that satisfactory results are only to be obtained by careful regulation of these and other factors. In a recent paper Goebel [1927] states that the oxidation of glucose is more complete if the alkali be added gradually over a period of several minutes. In all these investigations the estimations were carried out for the most part on relatively large amounts of the sugars. As we wished to apply the method to the estimation of quantities of the order of 1 mg. it seemed advisable to study first the effect of the various factors under the conditions involved in such micro-estimations.

The following is the general procedure adopted for these tests.

1 or 2 cc. of the sugar solution (0·1 %) was measured into a 50 cc. Erlenmeyer flask, followed by 3 cc. of 0·02 *N* iodine and distilled water to make up the volume to 6 cc., this last serving to rinse down the sides of the flask. The alkali solution was then added slowly and the flask immediately closed with a rubber stopper and immersed in a water-bath at the specified temperature. The use of "room temperature" was given up as the variations were found to be sufficient to produce irregular results. At the end of the given time the contents of the flask were acidified with 1 cc. 0·5 *N* H_2SO_4 and titrated with 0·005 *N* sodium thiosulphate using as indicator 3 drops of a 1 % solution of soluble starch in a saturated solution of sodium chloride. All estimations were made in duplicate and blank tests, also in duplicate, were simultaneously carried out, using distilled water in place of the sugar solutions. These blanks were further checked by direct titration of 3 cc. of the iodine solution with the thiosulphate. The difference between the blanks and the direct titration did not as a rule exceed 0·1 cc., and was due to loss of iodine by volatilisation, impurities in the alkali, etc. The usual precautions were taken as regards calibration of the Ostwald pipettes and the burette. The sugars used in these tests gave values for the specific rotation and reducing power by the Hagedorn-Jensen method as shown in Table I. The values for the reducing power are given in terms of glucose and for the other sugars are of limited significance, but the very low value for galactose, which is similar to that recorded by Pucher and Finch [1928], is of interest in relation to the results obtained by the iodimetric method.

The results given in Tables II and III show the influence of varying quantities of sodium hydroxide and sodium carbonate respectively on the oxidation of glucose and fructose.

Table I.

Sugar	$[\alpha]^{20°}_{5461}$ $c=10\%$	Reducing power %
Glucose (Kahlbaum)	+ 62·8° (21°)	99·9
Fructose (Kerfoot)	− 108·7°	96·2
Galactose (Kahlbaum)	+ 96·9°	73·0
Lactose	+ 64·6° (19°)	67·0
Maltose hydrate (Kahlbaum)	+ 154·8°	68·5
Sucrose	+ 78·6°	—

Table II.

Alkali: 0·1 N NaOH, in amount varying from 1 to 1·66 times the equivalent of iodine.

Vol. of 0·1 N NaOH cc.	Temp.	Time (mins.)	Glucose (1 mg.)	Glucose (2 mg.)	Fructose (1 mg.)
0·6	17°	20	− 0·5	− 8·7	3·2
			− 0·5	− 8·7	3·2
0·75	,,	,,	− 0·5	− 13·0	3·6
			− 1·0	− 8·7	3·6
0·9	,,	,,	+ 0·5	− 2·2	4·0
			+ 0·5	− 10·0	4·0
1·0	,,	,,	+ 0·5	− 2·2	4·0
			0	− 5·7	4·0
0·6	21°	5	− 15·7	—	0·4
			− 15·7	—	1·2
0·75	,,	,,	− 20·9	—	0·8
			− 13·1	—	0·8
0·9	,,	,,	− 12·1	—	0·8
			− 13·1	—	0·8
1·0	,,	,,	− 12·2	—	1·2
			− 12·2	—	1·2
0·6	21°	20	− 0·5	− 11·1	5·9
			+ 1·2	− 11·1	7·5
0·75	,,	,,	− 0·5	− 13·7	7·5
			− 0·5	− 13·7	10·0
0·9	,,	,,	+ 0·9	− 2·4	8·8
			− 1·3	− 2·0	8·8
1·0	,,	,,	+ 0·4	− 1·3	9·2
			− 1·3	− 0·9	9·2

For glucose the results are stated as percentage deviations from the theoretical values for complete oxidation to gluconic acid.

For fructose the results are stated as the percentage oxidised, calculated on the basis of the theoretical value for glucose.

The amount of iodine used was in all cases 3 cc. 0·02 N solution, equivalent to 5·4 mg. glucose. The titration thus required (for the blank) about 12 cc. 0·005 N thiosulphate. 2·25 cc. is equivalent to 1 mg. glucose. The results, though calculated to the first decimal place, are only significant to about 0·5 %.

These results show that:

(1) With sodium hydroxide (0·9 cc.) in the proportion of 3 mols. to 2 mols. of iodine, as recommended by Willstätter and Schudel, the oxidation of 1 mg. glucose is complete in 20 minutes at 17° or 21°, but not in 5 minutes at 21° (iodine in 5-fold excess). With 2 mg. glucose the results for 17° are irregular but are more satisfactory for 20 minutes at 21°.

Table III.

Alkali: 5 % Na_2CO_3 solution (0·94 N).

Vol. of 5 % Na_2CO_3 cc.	Temp.	Time (mins.)		Glucose 1 mg.	Glucose 2 mg.	Glucose 3 mg.	Fructose 1 mg.	Fructose 2 mg.	Fructose 3 mg.
0·1	21°	30		+0·4	−23·1	−61·7	—	—	—
,,	,,	,,		0	−33·3	−57·5	—	—	—
,,	,,	,,		−4·1	—	—	—	—	—
,,	,,	,,		−9·8	—	—	—	—	—
			Av.	−4·5	−28·2	−59·6			
0·2	21°	30		+1·0	−1·2	−18·5	0·4	0·8	1·4
,,	,,	,,		+1·0	−1·2	−23·4	0·4	2·5	2·2
,,	,,	,,		−1·5	−3·1	−33·3	1·7	—	—
,,	,,	,,		−2·4	−3·1	−33·3	0·9	—	—
,,	,,	,,		−0·2	—	—	—	—	—
,,	,,	,,		−0·2	−2·2	—	—	—	—
,,	,,	,,		−0·2	—	—	—	—	—
,,	,,	,,		−0·2	−2·2	—	—	—	—
,,	,,	,,		+2·3	—	—	—	—	—
,,	,,	,,		+2·3	—	—	—	—	—
,,	,,	,,		+2·6	—	—	—	—	—
,,	,,	,,		+2·6	—	—	—	—	—
			Av.	+0·6	−2·2	−27·1	0·8	1·6	1·8
0·4	21°	30		+0·7	−1·7	−10·3	0·4	0	—
,,	,,	,,		+0·7	—	—	0·4	0	—
,,	,,	,,		+0·7	−3·0	−8·4	0	—	—
,,	,,	,,		−2·0	—	—	0	—	—
,,	,,	,,		−1·5	−1·8	−11·6	0·4	—	—
,,	,,	,,		+0·3	—	—	—	—	—
,,	,,	,,		+0·7	−0·7	—	0·4	—	—
			Av.	+0·1	−1·8	−10·1	0·3	0	—
0·4	25°	30					0	0·7	—
,,	,,	,,		+0·3	−2·4	−8·3	0	0·4	—
,,	,,	,,		+0·7	−2·2	−7·9	0·4	—	—
,,	,,	,,		0	—	—	0·4	—	—
,,	,,	,,		0	—	—	0	—	—
,,	,,	,,		0	—	—	0	—	—
,,	,,	,,		0	—	—			
			Av.	+0·2	−2·3	−8·1	0·1	0·5	—
0·8	21°	30		−8·8	—	—	—	—	—
,,	,,	,,		−6·7	−20·3	−30·8	0·9	0·9	—
,,	,,	,,		−8·8	—	—	—	—	—
,,	,,	,,		−8·8	—	—	—	—	—
,,	,,	,,		−9·7	−20·5	—	2·6	0·9	—
			Av.	−8·6	−20·4	−30·8	1·7	0·9	—

(2) Under the conditions necessary for the complete oxidation of 1 mg. glucose, fructose is also oxidised to a significant extent, which may amount to 9 % in 20 minutes at 21°. This disadvantage is not overcome by using other proportions of sodium hydroxide.

(3) With sodium carbonate in suitable amounts (0·2 cc. or 0·4 cc. 5 % Na_2CO_3), oxidation of 1 mg. glucose is complete in 30 minutes at 21°; 2 mg. glucose are oxidised to the extent of 98 %, but with larger amounts the results are low and irregular. Fructose is oxidised only to a very small extent, the values shown (0–2 %) lying close to the limits of titration errors by this method.

(4) With amounts of 5 % sodium carbonate less than 0·2 cc. or more than 0·4 cc. the oxidation of glucose is incomplete.

From other tests it was found that variations in the temperature between 21° and 25°, or in the time between 20 and 45 minutes did not affect the results. The use of sodium carbonate therefore offers a greater latitude as regards amount of alkali, temperature etc. than is permissible with sodium hydroxide.

For routine estimations the conditions adopted were, for amounts of sugar equivalent to 1–1·5 mg. glucose, 3 cc. 0·02 N iodine; 0·2 cc. 5 % Na_2CO_3; 21° and 30 minutes. These correspond closely with the conditions recommended by Bougault for larger quantities of sugar.

Table IV gives the average results obtained with various sugars under the above conditions. For the aldoses the results are stated as percentages of the respective compounds calculated from the iodine reduced, according to the equation. For the ketoses and sucrose the results are given in terms of the equivalent quantity of glucose. The results for galactose were persistently low and were not improved by increasing the time or by adding the alkali over a period of 6 minutes[1]. In this table are also included values obtained for barium glucosemonophosphate, barium fructosemonophosphate (Neuberg) and barium fructosediphosphate, those for the fructose derivatives being calculated as percentage glucose. In spite of the low value for the glucosemonophosphate there is good reason for believing that the specimen was free from fructosemonophosphate (Robison and King, unpublished work).

It should perhaps be emphasised that great caution is necessary in interpreting the results obtained by this method with crude biochemical products since many compounds other than aldoses may react with iodine under these conditions.

Table IV.

Sugar	1 mg.	2 mg.	3 mg.	4 mg.
Glucose	100·6	97·8	78·1	—
Galactose	97·0	96·5	85·6	—
Maltose	102·8	100·9	—	78·4
Lactose	102·3	98·3	—	75·5
Fructose	0·8	1·6	1·8	—
Sucrose	2·0	0·8	1·7	—
Barium glucosemonophosphate	—	90·2	—	—
Barium fructosemonophosphate	—	—	—	(15 mg.) 3·0
Barium fructosediphosphate ...	—	—	—	0–2·0

Some experiments were carried out to determine to what extent the Lobry de Bruyn and van Ekenstein transformation may account for the high values obtained for fructose using sodium hydroxide. In order to increase the effect, the ratio of sodium hydroxide to iodine was raised to 10 : 3 but the concentration of alkali was only 0·03 N. The results are set out in Table V.

[1] *Note* (*added 7th June* 1929). Satisfactory results have, however, since been obtained with galactose, prepared by the hydrolysis of a highly purified specimen of α-methyl-d-galactoside, for which we have to thank Mr J. A. Pryde.

Column A. Reducing power of fructose by the usual carbonate method (in this case, 0·4 cc. 5 % Na_2CO_3 was used) 30 minutes at the specified temperatures.

Column B. Reducing power using 2 cc. 0·1 *N* NaOH, 30 minutes at the specified temperature.

Column C. Fructose solution allowed to remain for 30 minutes at the specified temperature in presence of 2 cc. 0·1 *N* NaOH, then neutralised and reducing power estimated by the carbonate method (30 minutes, 21°).

Column D. Fructose solution allowed to remain for 30 minutes at the specified temperature in presence of 2 cc. 0·1 *N* NaOH. Iodine then added and solution left for further 30 minutes at the specified temperature.

Table V.

Amount of fructose mg.	Temp.	*A*	*B*	*C*	*D*
1	21°	0·4	13·1	5·3	24·9
5	21°	0·6	16·8	4·4	16·9
1	25°	0·4	17·8	11·4	25·8
5	25°	0·9	17·2	6·2	22·2
1	38°	4·0	68·0	22·7	84·1
5	38°	2·5	30·4	14·7	39·4

Comparison of columns *A* and *C*, as also of columns *B* and *D*, shows that some change is produced by the action of the alkali alone, but this could at most account for only a fraction (30–40 %) of the total oxidation effected by the combined action of the iodine and alkali, shown in column *B*, unless the nett rate of the transformation of fructose into glucose is greatly increased in presence of the iodine owing to the rapid removal of the aldose by oxidation.

Summary.

The conditions for the estimation of very small amounts of aldoses by the iodimetric method have been investigated. Satisfactory results are obtained using 3–4 times the theoretical quantity of iodine with sodium carbonate as the alkali, and allowing the oxidation to proceed during 30 minutes at 21°. Under these conditions the oxidation of glucose is complete while fructose and sucrose are only oxidised to a very small extent.

Values obtained for other sugars and for hexosemono- and hexosediphosphates are also given.

The extent to which fructose is oxidised by iodine in presence of excess of sodium hydroxide is much greater than can be explained by the Lobry de Bruyn and van Ekenstein transformation unless it is assumed that the change from ketose to aldose is largely increased owing to the rapid and continuous removal of the latter by oxidation.

We are greatly indebted to Mr J. L. Baker and Mr H. F. E. Hulton for kindly supplying us with several specimens of pure sugars.

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LX. THE CHEMICAL CONSTITUTION OF THE GUMS.

PART I. THE NATURE OF GUM ARABIC AND THE BIOCHEMICAL CLASSIFICATION OF THE GUMS.

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Preamble.

The latest work which has been carried out on gums is not of very recent date, and advances in carbohydrate chemistry which have been made since that time make it desirable that this work should be repeated. The physical difficulties of working with these substances are considerable, and the situation is aggravated by the fact that the natural gums are frequently composed of mixtures of several individual types. Separation of such mixtures is very difficult, and it is probable that physical methods based on their colloidal properties will have to be elaborated.

Early investigators have shown that the hydrogen and oxygen in gums exist in approximately the same proportions as in water, and accordingly the gums were considered to be true carbohydrates. Later, by the action of acids, various sugars were obtained from them by hydrolysis, and the gums were thereupon classified as polysaccharides. Still later, it was demonstrated that they are not of a true polysaccharide nature, since they consist of numbers of sugar molecules united to a central nucleus of a sugar acid nature. The attachment seems to be of an ethereal or glucosidic type, and not an ester type, since the gums retain acidic properties, and are capable of forming salts. In point of fact, they are usually found as salts of calcium, magnesium, or potassium.

It is to be emphasised that the classification of a substance as a gum is as yet mainly an arbitrary one, not being based on any constitutional knowledge, but decided by its origin and physical behaviour, together with the fact that it is of a carbohydrate nature. When the chemical composition of these substances has been determined, it may be possible to suggest a more rational grouping, and also to speculate with some degree of probability as to their rôle in nature.

The first member of this group to be studied was gum arabic, since from the work of previous investigators this appears to be a single substance.

THE COMPOSITION OF GUM ARABIC.

Introduction.

The early investigators of the composition of gum arabic seem chiefly to have been concerned in attempting to assign to it an empirical formula, and several workers in the first half of the last century gave analyses closely approximating to $(C_6H_{10}O_5)_n$. Neubauer [1854, 1857, 1863] seems to have been the first to record the fact that gum arabic is an acid. He prepared pure salts of this acid, and subjected them to analysis, from the results of which he deduced various empirical formulae for the salts.

O'Sullivan [1884] described some very careful and detailed investigations into the nature of this substance. His methods were, briefly, hydrolysis with acid, preparation of the barium salt of the hydrolysis product, fractional precipitation with alcohol, if necessary, and ultimate analysis of the salt obtained. By these means he concluded that this gum had an empirical formula of $C_{89}H_{142}O_{74}$ and consisted of molecules of four, or possibly five, hexose sugars, termed by him α-, β-, γ-, and δ-arabinoses, linked to a nucleus of a comparatively stable nature to which he assigned the empirical formula of $C_{23}H_{38}O_{22}$, and the name "arabinosic acid." In a later paper, O'Sullivan [1889] recognised the presence of only two sugars in this gum, a pentose, arabinose, and a hexose, galactose, and suggested a more extended formula, which will be quoted later.

Experimental.

A good commercial sample of gum arabic is a light transparent substance, slightly yellowish in colour, in droplets with a vitreous fracture. The ash content varies from 3 to 4 % or more, and there is some moisture present. This gum is found naturally as the salts of calcium, potassium, and magnesium. Pure acid gum arabic is prepared from the natural product according to the method employed by Neubauer [1854].

The gum is dissolved in warm water to give a fairly concentrated solution, which is then made acid by the addition of hydrochloric acid. Insoluble particles are allowed to settle, and the clear liquid is decanted off. The acid gum arabic is then precipitated by the addition of alcohol. Precipitation takes place when the alcohol content is as low as 50 %, but as a general rule this should be raised to 60 % so that precipitation may be complete. The acid gum arabic appears as a thick and curdy precipitate. This is filtered off, re-dissolved in warm water and acidified by the addition of a little hydrochloric acid just previous to precipitation with alcohol as before. This process is repeated at least three times. The final product is dried in absolute alcohol, and in a desiccator over phosphorus pentoxide. By this means an extremely pure product is obtained, the ash content of which is very low.

It was considered likely that the nucleus-acid of gum arabic, termed by O'Sullivan first "arabinosic acid" and later "arabic acid," might be of the

nature of a "uronic acid." Accordingly the yield of carbon dioxide on boiling with 12 % hydrochloric acid was determined in the same way as for pectin products [Nanji, Paton and Ling, 1925].

Acid gum arabic. Sample I.	Ash	0·26 %
Yield of CO_2 on an ash-free basis	(i)	3·11 %
	(ii)	3·17 %
	Mean	3·14 %
Corresponding to uronic acid anhydride		12·56 %

Since this gum has been shown by many workers to contain pentose, the furfuraldehyde yield was determined by distillation and precipitation as phloroglucide. The precipitated phloroglucide was in each case extracted with absolute alcohol, since the presence of methylpentose in this gum has been suggested. Although by this extraction a considerable amount of substance was removed, concurrent figures were never obtained. Further the alcoholic extract was never of a reddish colour, as it is stated to be in the presence of the phloroglucide of methylfurfuraldehyde, but always a deep green. The substance removed is probably the phloroglucide of ω-hydroxymethylfurfuraldehyde, obtained in small quantities from the hexosan units.

Acid gum arabic. Sample I.		
Yield of furfuraldehyde on ash-free basis	(i)	16·45 %
	(ii)	16·01 %
	(iii)	16·25 %
	Mean	16·24 %

Now it is known that the uronic acid anhydride units yield 16·66 % of their weight of furfuraldehyde, and consequently the 12·56 % of uronic acid anhydride which is present in this sample of acid gum arabic accounts for 2·09 % furfuraldehyde; the balance of furfuraldehyde, 14·15 %, being due to pentose.

Many workers have stated that the sugars to be obtained by the hydrolysis of this gum are arabinose and galactose. This statement was confirmed. It is not necessary to describe in detail the procedure, but it is sufficient to say that the characteristic osazones of arabinose and galactose were obtained from the hydrolysis liquid after removal of the acid. Arabinose diphenylhydrazone was prepared by treatment of the syrup with excess of diphenylhydrazine in alcoholic solution according to the method of Neuberg [1900]. The recrystallised hydrazone had a melting point of 202° (Neuberg: 204°). Mucic acid was also obtained by treatment with nitric acid. No trace of xylose could be detected by use of Bertrand's cadmium xylonobromide reaction; neither was any hexose other than galactose traced. It seems, then, that the only sugars formed on hydrolysis are arabinose and galactose.

It is clear then that the furfuraldehyde in the molecule due to pentose may be calculated as arabinose, indicating that there is present 26·31 % anhydro-arabinose ($C_5H_8O_4$), yielding on hydrolysis 29·89 % of arabinose.

No satisfactory quantitative method has ever been described for the estimation of galactose, the oxidation by nitric acid to mucic acid giving invariably a low result, the production of mucic acid being accompanied also by the formation of oxalic acid.

It is only possible, therefore, to arrive at figures for galactose by difference.

Acid gum arabic. Sample I.

Uronic acid anhydride	12·56 %
Anhydro-arabinose	26·31 %
Anhydro-galactose (by difference)	61·13 %

yielding on hydrolysis, arabinose 29·89 % and galactose 67·92 %.

Much of the work of O'Sullivan is based on the estimation of the barium content of the barium salt, and although this undoubtedly gives a fair indication of the carboxyl groups present, it does not seem that it can be taken as an absolute figure, unlike the determination of carbon dioxide yield in this work. The method followed by him in the preparation of the barium salt of acid gum arabic was exactly to neutralise a solution of the acid with baryta water, and then to precipitate with alcohol, and dry. Since the acid is a very weak one, it is difficult to determine the precise neutral point. The alcohol precipitate is of a bulky nature, and would undoubtedly carry down with it any excess baryta which might be present, and so vitiate the results. Alternatively, if the end-point were not quite reached, the results might be low owing to the presence of free acid gum arabic.

The barium salt of acid gum arabic was prepared by careful addition of baryta as described by O'Sullivan. The amount of baryta to be added was ascertained on a duplicate, using phenolphthalein as indicator. After filtration, the salt was precipitated with alcohol, washed, and dried. A barium estimation was carried out on this preparation, gravimetrically as sulphate.

Barium content of salt of acid gum arabic.

Preparation	(i)	5·07 % Ba
,,	(ii)	5·27 % Ba

Now, the yield of carbon dioxide from the preparation indicates the presence of carboxyl groups which would only be capable of uniting with 4·89 % barium. There is the possibility, of course, that there might be present in the nucleus of this gum some simple organic acid, not of a uronic type, not yielding carbon dioxide under the conditions of that estimation. This, however, is very unlikely, since no such acid has been discovered in the hydrolysis liquid, and further, the difference between the theoretical barium content and that actually found is only small, and is to be anticipated from the method of preparation. It seems, therefore, that this particular method, the preparation of the barium salt and the subsequent estimation of its barium content, is not reliable as giving an indication of the amount of carboxyl groups present. Very many of O'Sullivan's conclusions are based upon the results

of this determination, and consequently they cannot be accepted as absolute figures, though, as seen above, it is likely that they are fair approximations.

O'Sullivan in his earlier paper on this subject considered all the sugars split off to be of a hexose nature, but after the discovery of the pentose nature of arabinose he assigned to acid gum arabic the following formula:

$$2(C_{10}H_{16}O_8) . 4(C_{12}H_{20}O_{10}) . (C_{23}H_{30}O_{18}).$$

He described it as "di-arabinan-tetra-galactan-arabic acid," the term "arabic acid" being employed for the nucleus-acid in place of the term "arabinosic acid" employed by him previously. It is necessary to note that the "arabic acid" of other workers corresponds with that which, to prevent confusion, is described in this paper as "acid gum arabic." In the formula above "arabinan" and "galactan" respectively stand for two molecules of arabinose and galactose less two molecules of water.

By calculation, such a molecule consists of

anhydro-arabinose $(C_5H_8O_4)_n$	21·84 %
anhydro-galactose $(C_6H_{10}O_5)_m$	53·60 %
anhydro-"arabic acid"	24·56 %

yielding on hydrolysis, arabinose 24·82 % and galactose 59·57 %.

It will be seen that these figures differ very considerably from those obtained experimentally in this paper, the difference between the anhydro-"arabic acid" of O'Sullivan's formula and the uronic acid anhydride of the preparation being particularly marked. On the other hand, it must not be assumed that all samples of gum arabic are identical in composition. Such is quite definitely not the case, though they appear to be similarly constituted. This means, however, that it is not possible to assign to this substance any definite empirical formula, but only to indicate the constituents and the probable method of linkage.

Sample II of gum arabic, treated as before to obtain the acid gum arabic, gave on analysis the following figures:

Acid gum arabic. Sample II. Ash 0·24 %.

Furfuraldehyde yield on ash-free basis	(i)	14·00 %
	(ii)	13·86 %
	(iii)	13·98 %
	(iv)	13·89 %
	Mean	13·93 %
Yield of carbon dioxide on ash-free basis	(i)	4·36 %
	(ii)	4·42 %
	Mean	4·39 %

By calculation

uronic acid anhydride	17·56 %
furfuraldehyde due to uronic acid anhydride	2·91 %
balance of furfuraldehyde due to pentose	11·02 %

The figures for Sample I, and those obtained by calculation from O'Sullivan's formula, are quoted for comparison.

	Sample I	Sample II	O'Sullivan
Uronic acid anhydride	12·56	17·56	(anhydro-"arabic acid" 24·56)
Anhydro-arabinose	26·31	20·52	21·84
Anhydro-galactose	61·13	61·92	53·60
Yielding on hydrolysis:			
Arabinose	29·89	23·32	24·82
Galactose	67·92	68·80	59·57

By hydrolysis with acids O'Sullivan was able to arrive at conclusions stated to justify his formula. He was, however, largely concerned with the identification of the sugars liberated, and the barium content of the salts of the acid residues isolated. He appears to have chosen concentrations of sulphuric acid for hydrolysis in a rather haphazard way. In the work described here, it was thought desirable to adhere throughout to one concentration, namely, 3 %. The hydrolysis was carried out by O'Sullivan in the following manner. To the acid gum arabic in solution was added the necessary amount of sulphuric acid, and the whole boiled. At the conclusion of the period of hydrolysis the solution was cooled, and neutralised with a hot solution of baryta. The precipitated sulphate was filtered off, and the filtrate evaporated and poured into excess of alcohol. The precipitate was repeatedly dissolved in water and reprecipitated by alcohol.

This method was followed for a time, with the difference that carbon dioxide was passed through after the addition of the baryta to remove any excess. It was found, however, that by this method it was not possible entirely to free the hydrolysis product from the sugars liberated at the same time, and as a result sticky or slimy precipitates were obtained. Also the ash content of those that were obtained in a reasonably friable condition was unduly high.

Accordingly the method was modified. At the close of the period of hydrolysis, while still very hot, the sulphuric acid employed is nearly neutralised by the addition of solid barium carbonate in a very fine condition. After standing for a few moments, the precipitated sulphate is filtered off. Immediately, while the filtrate is still hot, boiling alcohol is poured in until the concentration of alcohol reaches 60 %. The precipitate which forms settles quickly, and the supernatant liquid is poured off before it has cooled. Since hot alcohol of a concentration of 60 % does not precipitate either galactose or arabinose, the end-product obtained by this means is free from these substances. The precipitate is dissolved in water, filtered, heated nearly to boiling and reprecipitated by hot alcohol as before. This is repeated several times. The final product obtained is not sticky, and on being brought into contact with absolute alcohol it becomes white and friable. It will be noticed that by this method the free acid hydrolysis product is obtained in place of the barium salt as by the method of O'Sullivan.

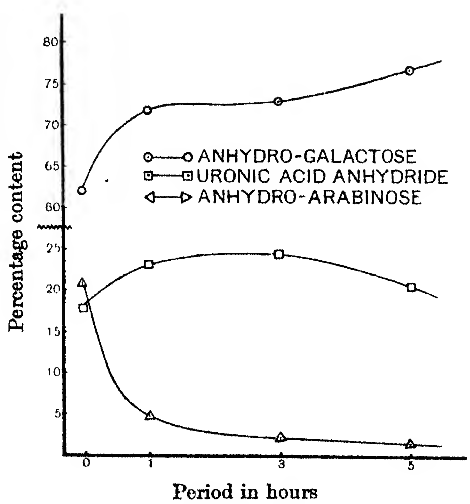
Hydrolyses were carried out for periods of 1, 3, 5, 6, 7 and 10 hours with

3 % sulphuric acid. It is inadvisable to commence with less than 200 g. in each case for the yields are small. Three times the weight of sulphuric acid was employed. No substance remained precipitable by hot alcohol after either 10 or 7 hours' hydrolysis. Only traces precipitable by alcohol were found after 6 hours, the quantity being quite insufficient for analysis.

The following were the analytical figures obtained, in each case being the mean of two or more closely agreeing determinations. All hydrolyses were carried out upon Sample II of gum arabic.

	Acid gum arabic Sample II	Product after 1 hour's hydrolysis	Product after 3 hours' hydrolysis	Product after 5 hours' hydrolysis
	%	%	%	%
Ash	0·24	2·87	3·32	3·91
Furfuraldehyde yield (ash-free) ...	13·93	6·48	5·22	4·28
CO_2 yield (ash-free)	4·39	5·79	6·13	5·25
Uronic acid anhydride	17·56	23·16	24·52	21·00
Furfuraldehdye due to uronic acid	2·91	3·86	4·08	3·49
Balance of furfuraldehyde	11·02	2·62	1·14	0·79
Anhydro-arabinose	20·52	4·93	2·12	1·48
Anhydro-galactose	61·92	71·91	73 36	77·52
Yielding arabinose	23·32	5·60	2·41	1·68
Yielding galactose	68·80	79·91	81·53	86·14

From a consideration of these figures it is possible to obtain some insight into the composition of this gum. The results are even clearer when plotted as a graph. It will be seen from the graph that the arabinose is rapidly removed from the molecule during the first hour of hydrolysis, and the amounts of uronic acid anhydride and of anhydro-galactose rise sharply, proportionately,



Hydrolysis of gum arabic with 3 % sulphuric acid

and approximately evenly. During the second and third hours this condition is maintained at a much slower rate, and by this time practically all the arabinose has been removed. After the third hour it is seen that the uronic acid anhydride tends to diminish a little, and the anhydrogalactose to rise.

It is quite evident, therefore, that the linkage of the arabinose units is different in nature from that between the other units, since it is very much weaker, being ruptured by brief treatment with acid. Knowing that gum arabic is not a reducing body it is probable that the linkage between the arabinose units and the nucleus-acid is an ethereal or glucosidic linkage.

It would seem that the nucleus-acid, the "arabic acid" of O'Sullivan, is composed of galactose and the uronic acid, probably in the proportion of two or three molecules of the former to one of the latter, or some multiple of these figures. This is a little surprising, but even more unusual is the fact that the galactose does not appear to be split off except when the nucleus-acid is completely ruptured. That this must take place continuously during the hydrolysis is evidenced by the fact that galactose in some quantity can be obtained from the hydrolysis liquid after less than 3 hours' hydrolysis. Nevertheless the uronic groups are apparently removed somewhat more easily than the galactose units. The nature of the uronic acid has not been definitely established. It is, however, in all probability, galacturonic acid, since glycuronic acid, although excreted in certain abnormal conditions of animal metabolism, has never been unquestionably proved to be a constituent of plant materials, save possibly in the hemicellulose A isolated by O'Dwyer [1926] from beechwood.

Discussion.

Work which has been carried out on other gums has shown that they also are constructed in much the same general way as gum arabic, namely by linkage of hexose or pentose sugars with uronic acid units. It is possible, therefore, tentatively to suggest a position for the gums in the general field of plant biochemistry. Candlin and Schryver [1928] have suggested the use of the word "polyuronide" to describe a substance which consists of sugars linked to a uronic acid. It is evident, then, that the gums may be described as "polyuronides" if this term be accepted.

Table I. *Uronic acid anhydride content of some plant products.*

Substance	Source	Uronic content %	Sugars present	Authority
Pectic acid	Lemons	70 56	Galactose, arabinose	Nanji, Paton and Ling [1925]
Hemicellulose B	Beechwood	64·0	Galactose, arabinose	O'Dwyer [1926]
Mild oxidation products of pectin	Lemon pectin	60–55	Galactose, arabinose	Norman (unpublished)
Tragacanthin	Gum tragacanth	50·8	Arabinose	Norman (unpublished)
Hemicellulose by alkali treatment	Citrus pectin	37·3	Presumably galactose, arabinose	Candlin and Schryver [1928]
Hemicellulose B	Oats straw	31·8	? ?	Norman (unpublished)
Bassorin	Gum tragacanth	30·0	Arabinose, galactose, xylose?	Norman (unpublished)
Hemicellulose by alkali treatment	Onion pectin	21·5	Presumably galactose, arabinose	Candlin and Schryver [1928]
Acid gum arabic	(Sample II)	17·56	Galactose, arabinose	Norman
Hemicellulose B	Beechwood	18·8	? ?	Candlin and Schryver [1928]
Hemicellulose A	Turnip	15·4	? ?	Candlin and Schryver [1928]
Acid.gum arabic	(Sample I)	12·56	Galactose, arabinose	Norman
Hemicelluose A	Beechwood	11·5	? ?	Candlin and Schryver [1928]
Hemicellulose A	Beechwood	11·0	Xylose (galactose?)	O'Dwyer [1926]
Hemicellulose A	Oats straw	10·8	Arabinose ?	Norman (unpublished)
Hemicellulose B	Turnip	10·7	? ?	Candlin and Schryver [1928]

Several recent workers have shown that natural hemicelluloses consist of hexose and pentose sugars linked together and usually in combination with a uronic acid. There does not seem to be, therefore, any essential difference in general structure between the gums and the hemicelluloses, which is rather supported by the fact that they are not dissimilar in physical properties and solubilities. It is rapidly becoming clear that the uronic acids are much more widely distributed in vegetable materials than was previously supposed (see Table I[1]).

Candlin and Schryver [1928] have recently demonstrated that pectin may be decarboxylated by treatment with alkali, the degree of decarboxylation depending upon the concentration and temperature of the alkali, with the resulting production of substances of a hemicellulose type, resembling in all respects the hemicelluloses isolated directly from wood. It would seem, then, not unlikely that in nature both hemicelluloses and gums may be produced by partial decarboxylation of pectin. This change may be brought about *in vitro* not only by alkali treatment but also by mild oxidation, on which point details of some work will shortly be published. Such a process as mild oxidation more nearly approaches, of course, the changes which may possibly take place in the living plant.

Both von Fellenberg [1918] and Ehrlich [1927] have suggested the possibility that the pectic substances may undergo transformation to lignin, and O'Dwyer [1928] states that it is not unlikely that hemicellulose may be a transition product. She showed [1926] that unlignified tissues contain a much larger amount of pectin than of hemicelluloses, while in lignified tissues the opposite holds, in fact only the merest traces of pectin are usually present. Candlin and Schryver [1928] repeated the suggestion that lignin may be derived from pectin, but their experimental work, which resulted in the artificial production of a hemicellulose from pectin, did not lend support to this theory. It must be admitted that it makes an attractive theory to couple the two substances, pectin and lignin, since the observation that pectin decreases on lignification seems to hold good generally, but nevertheless on purely chemical and constitutional grounds it is not at present easy to see how the transformation may take place. Further, the actual amount of lignin present in lignified tissue is very considerably in excess of the amount of pectin which was present in the same tissue before lignification. On quantitative grounds also, there is considerable support for the view that pectin is converted into hemicellulose as the tissue becomes older. Though this process may be coincident with the development of lignification, it is not possible from present evidence to assume any interrelation.

On purely chemical grounds it is possible to account for the formation of substances of the nature of pectin, hemicelluloses and gums, by the protracted

[1] It is probable, if not certain, that several of the products mentioned in this table, notably the hemicelluloses, are mixtures, and if suitable means of separating them can be elaborated even more significant uronic figures may be obtained.

mild oxidation of linked hexose units. For example, when *d*-glucose is linked in some form as a polysaccharide through its carbonyl groups, mild oxidation will have the effect of converting the terminal carbinol groups to carboxyl, and so, in a simple hypothetical case, forming a conjugated *d*-glycuronic acid, which on complete decarboxylation yields a xylan: similarly *d*-galactose might be supposed to give rise to an araban. If oxidation is only partial, that is to say, if some units of the hexosan are incompletely oxidised, then other complex substances containing hexose, uronic and pentose groups would be formed, akin in all respects to pectin, hemicelluloses and gums. It is undoubtedly a fact that it is the derivatives of galactose, possibly formed in this way, which are met with most frequently in the substances classed as hemicelluloses and gums, and further, each of the units in pectin is derivable from galactose. Xylans and gluco-xylans are relatively less common and widespread than the galacto-arabans. It is curious that galactose thus appears to occupy a place rather more important in this transition to pentosans than does glucose, while it is glucose which is the most widespread unit of hexosans, *e.g.* in starch and cellulose. The change from glucose to galactose is one which must take place readily in plants, and also in animals, as evidenced by the production of lactose on a diet free from galactose. The explanation of this prominence of galactose and its derivatives in plant constituents is not obvious, unless it be that hexose in the form of galactose is less readily respired than as glucose.

It would seem that the first apparent stage in the oxidation of galactans is the production of pectin, and having regard to the flexibility of the process of mild oxidation, it is perhaps remarkable that a substance of such definite character as pectin should appear. The homogeneity of pectin preparations from many sources appears to have been established by the work of Clayson, Norris and Schryver [1921], and of Norris and Schryver [1925]. Recently, however, Ehrlich and Schubert [1926] have claimed that pectin from flax contains xylose, in addition to galactose, galacturonic acid, and arabinose. Henderson [1928], on rather insufficient evidence, has claimed, on the contrary, that pectin from flax contains only galactose and galacturonic acid. In view of this conflict of opinions this form of pectin was re-investigated by Norris [1929], who finds no reason for departing from the original six ring formula put forward by Nanji, Paton and Ling [1925].

The formation of pectin in the plant appears to take place when metabolism is at its highest peak, that is when growth is most rapid. As growth slows down and maturity is reached, so does the production of pectin decrease, and that present is slowly converted into other substances, probably hemicelluloses and gums. It seems likely, if not certain, that the hemicelluloses and gums are not substances definite in composition but are built up on a general plan from the intermediate oxidation products of hexosans. If the oxidation is prolonged, it may be that complete decarboxylation follows, so that true pentosans would be formed. It must be admitted, however, that

it has yet to be demonstrated that such substances as true pentosans have any existence in fact. Examinations of preparations hitherto regarded as being true pentosans have shown that a small quantity of uronic acid is present, and this, it is claimed, is the clue to their formation.

Summary.

1. It is shown that the acid group in gum arabic is of the uronic type.
2. A method of isolation of the acid hydrolysis products is given.
3. The statement that the only sugars present in gum arabic are galactose and arabinose is confirmed; analytical figures are given for these substances, both in the untreated gum and in the hydrolysis products.
4. It is clear that gum arabic is not a substance of definite empirical formula. It is possible, however, to indicate its general composition—a nucleus-acid consisting of galactose and a uronic acid, probably galacturonic acid, to which is linked arabinose by glucosidic linkages. The arabinose is in consequence more easily split off than the other components.
5. There seems to be no essential difference in structure between gums and hemicelluloses, both consisting of hexose and pentose sugars linked to uronic acids.
6. It is suggested that it is by the protracted mild oxidation of linked hexose, and particularly galactose units, that pectin and the hemicelluloses and gums are formed.

In conclusion it is a pleasure to express my thanks to Prof. A. R. Ling and Dr F. W. Norris for their assistance and advice.

Addendum (June 8th, 1929). While the above communication was in the press, Butler and Cretcher (*J. Amer. Chem. Soc.* 1929, **51**, 1519) have published some work on the constitution of this gum. They claim to have isolated an aldobionic acid, galactose-glucuronic acid, corresponding to the nucleus-acid of O'Sullivan. To this are attached galactose, arabinose, and rhamnose. They do not appear to recognise the fact that gum arabic is not a specific substance. Further, it is not possible to agree that all the phloroglucide soluble in alcohol must be derived from methylpentose, for it is well known that under the conditions obtaining in this estimation, hexosan units yield a small but inconstant amount of ω-hydroxymethylfurfuraldehyde, the phloroglucide of which is soluble in alcohol. Accordingly the figure quoted by them for rhamnose is probably much too high. On the other hand it is not unlikely that that for galactose is too low, since the mucic acid method is not strictly quantitative.

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LXI. OBSERVATIONS ON THE CARBOHYDRATE METABOLISM OF TUMOURS.

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The studies of Warburg and his collaborators on the carbohydrate metabolism of surviving tumour tissues [1926] have dealt mainly with two transplantable strains of rat tumours, Jensen's rat sarcoma and Flexner's rat carcinoma, and the Rous chicken sarcoma.

On the basis of metabolism values found for a large number of these tumours, together with those obtained for a more limited series of human neoplasms, Warburg has suggested several generalisations, showing characteristic relationships between the magnitudes of the respiration and the aerobic and anaerobic glycolysis. The constant result which emerged was the abnormally high value of the anaerobic glycolysis as compared with the respiration.

Assuming that the oxygen utilised was functioning at its maximum efficiency in causing the removal or non-formation of lactic acid under aerobic conditions, the respiration was found inadequate to check the glycolysis completely, a relatively large excess fermentation remaining.

Representing this excess fermentation by U, the respiration by Q_{O_2}, and the anaerobic glycolysis by $Q_M^{N_2}$

$$U = Q_M^{N_2} - 2\,(Q_{O_2}).$$

Positive values for U were invariably found for the tumours quoted.

Aerobic glycolysis is not a specific feature of tumour tissue. Warburg [1929] has recently summarised examples of normal tissues possessing aerobic glycolysis, and Crabtree [1928] showed it to be a property of certain pathological overgrowths associated with intracellular viruses.

During the last year, as material has become available, the carbohydrate metabolism of several strains of transplantable mouse tumours, propagated in this laboratory, has been measured, and the results are collected in Table I. Although Warburg no longer adheres to the classification of tissues on the basis of a positive or negative value of U, throughout this communication the value will be introduced as affording an arbitrary standard of comparison, placing the present measurements in line with those previously recorded.

The manometric technique elaborated by Warburg has been followed throughout.

Table I. *Carbohydrate metabolism of mouse tumours.*

Tumour	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Meyerhof quotient	U
Crocker sarcoma	-10·6	+14·8	+30·4	1·5	+9·2
,,	-14·6	+15·6	+25·2	0·6	-4·0
,,	-30·9	+20·7	+31·7	0·4	-30·1
,,	-13·9	+15·0	+26·5	0·8	-1·3
,,	-8·2	+16·3	+26·5	1·2	+10·1
,,	-16·5	+16·2	+30·5	0·9	-2·5
,,	-7·6	+13·2	+30·6	2·3	+15·4
,,	-15·5	+17·3	+24·8	0·5	-6·2
,,	-24·4	+19·4	+24·9	0·2	-23·9
,,	-11·9	+16·7	+22·1	0·5	-1·7
,,	-6·5	+12·6	+23·2	1·6	+10·2
,,	-20·7	+19·7	+31·3	0·6	-10·1
,,	-21·4	+20·9	+33·3	0·6	-9·5
Tar carcinoma (Sp) 2146	-19·3	+19·6	+23·5	0·2	-15·1
,,	-21·3	+18·4	+24·5	0·3	-18·1
,,	-15·2	+14·3	+24·7	0·7	-5·7
,,	-31·0	+18·6	+25·7	0·2	-36·3
,,	-19·5	+11·6	+24·0	0·5	-15·0
,,	-13·3	+8·5	+24·0	0·9	-2·6
37 sarcoma	10·9	+12·6	+23·5	1·0	+1·7
,,	-9·8	+15·3	+29·4	1·5	+9·8
,,	-12·2	+6·5	+26·6	1·6	+2·2
,,	-12·1	+6·9	+27·6	1·6	+3·4
,,	-22·3	+14·4	+32·4	0·8	-12·2
,,	-24·3	+14·8	+33·4	0·7	-15·2
Tar sarcoma Bonné	-15·9	+9·1	+20·7	0·7	-11·1
,,	-13·9	+8·6	+20·8	1·0	-7·0
,,	-9·9	+12·3	+24·9	1·3	+5·1
Tar carcinoma 173	-12·0	+15·2	+30·7	1·3	+6·7
,,	-13·0	+16·3	+24·1	0·6	-1·9
,,	-20·1	+15·4	+31·9	0·8	-8·3
,,	-16·6	+20·7	+28·9	0·5	-4·3
,,	-17·0	+19·5	+28·9	0·5	-5·1
,,	-16·7	+17·0	+36·0	1·1	+2·6
,,	-14·4	+18·9	+37·0	1·2	+8·2
,,	-14·5	+10·2	+21·5	0·8	-7·5
,,	8·2	+6·7	+21·5	1·8	+5·1
,,	10·0	+8·2	+26·5	1·8	+6·5
,,	-18·3	+11·6	+27·5	0·8	-9·1
Sarcoma 2529	-14·8	+14·9	+32·6	1·2	+3·0
,,	-5·6	+12·8	+30·6	3·5	+19·4
,,	-16·5	+17·8	+36·3	1·1	+3·3
,,	-19·6	+22·1	+36·9	0·8	-2·3
,,	-16·0	+13·5	+28·3	0·9	-3·7
,,	-11·6	+12·7	+28·3	1·3	+5·1
,,	-10·9	+14·8	+29·7	1·4	+7·9
,,	-12·9	+15·1	+28·7	1·1	+2·9
Glycogen-carcinoma 113	-15·5	+6·8	+12·6	0·4	-18·4
,,	-12·5	+5·0	+12·7	0·6	-12·3
,,	-8·3	+3·4	+14·4	1·3	-2·2
,,	-7·6	+2·8	+14·1	1·5	-1·1
,,	-11·4	+4·3	+16·1	1·0	-6·7
Melanotic sarcoma	-5·6	+7·2	+23·7	2·9	+12·5
,,	-8·8	+4·3	+15·3	1·3	-2·3
,,	-7·7	+4·4	+15·1	1·4	-0·3
,,	-11·7	+7·0	+8·9	0·2	-14·5

The composition of the saline medium used, unless otherwise stated, was:

Salt	Moles per litre
NaCl	0·121
KCl	0·0025
$CaCl_2$	0·0018
$NaHCO_3$	0·025

Glucose was added in a concentration of 0·2 %, and the appropriate gas phase was 5 % CO_2 in O_2 or in N_2.

The method of expressing results is that used by Warburg and is explained in an earlier communication of the present author [1928].

Though every precaution was taken to ensure efficient metabolism, by using thin sections which permit perfect diffusion of metabolites, and by removal of all traces of necrosis visible to the naked eye, the results obtained were widely divergent in character.

The great variability in the absolute and relative magnitude of the metabolism values is not merely between the various strains investigated, but is apparent among different tumours of the same strain.

About one-third show values comparable to those found for Jensen's rat sarcoma and Flexner's rat carcinoma (see Table VI). The majority deviate from these standards, chiefly by exhibiting a higher respiration, both in its absolute value and also in its relation to the aerobic and anaerobic glycolysis. The relationship is emphasised by the negative value of U in numerous cases.

Many factors could conceivably operate in contributing to these abnormal findings: the generally more active metabolism of the mouse compared with larger animals, the variations in the environment at the site of growth, the possible fluctuations in the respiratory quotient, or the effectiveness of the blood supply. Also, in some tumour strains phases of rapid growth alternate with phases of depression with slow growth, and the position of the tumour in such a cycle at the time of the experiment would probably exert its influence.

Some of these possibilities were tested, and the results are recorded in later sections of this communication. Warburg postulates a disturbance of respiration as being the fundamental cause of the development of aerobic glycolysis. In many of the tumours included in the above table the respiration is very high, exceeding that of any tissue, normal or malignant, so far examined. In such cases, apparently, the Pasteur reaction fails to function, and until more is known of the mechanism by which respiration checks fermentation, the value of hypotheses involving conceptions of different kinds of respiration is doubtful.

The measurement of the respiratory quotient.

The Ringer solution used for the simultaneous measurement of respiration and aerobic glycolysis closely approximates, in its salt content and bicarbonate concentration, to physiological standards. The p_H is regulated by using 5 % CO_2 in O_2 as the gas phase, and for saturation of the media. The Henderson-Hasselbalch equation [Hasselbalch, 1917] then shows the p_H to be 7·35 at 37·5°. The procedure adopted for the measurement of the respiratory quotient involving absorption of CO_2 by potash is obviously inapplicable if these more accurate physiological conditions are to be maintained.

In the method utilised below, determinations of the respiratory quotient were made under the more favourable conditions used for simultaneous estimations of respiration and aerobic glycolysis.

Since Negelein [1925] has shown that the glycolysis effected by tumour tissue is a pure lactic acid fermentation and can be accurately measured by the fall in the bicarbonate concentration of the Ringer solution used experimentally, the respiratory CO_2 is obtained by deduction of the CO_2 evolved due to glycolysis (found by estimation of the bicarbonate concentration at the end of the metabolism experiment), from the total CO_2 evolved due to glycolysis and respiration and measured manometrically.

The following protocol will illustrate the method. In addition to the three manometers normally used for determination of aerobic metabolism, an extra one (Z) is required to measure the lowering of bicarbonate concentration during the 15 minutes' preliminary shaking until equilibrium is attained, *i.e.* to the time t°. This estimation, after correction for differences in weights of tissue used, serves as a control for the vessel Y in which the bicarbonate concentration is measured after 60 minutes' metabolism from the time t°. By rapid manipulation, the section of tissue can be removed from the Ringer solution in about 20 seconds, causing an error of no more than 0·5 %.

Protocol I.

Mouse tar carcinoma 173. Temp. 37·5°. Gas phase—5 % CO_2 in O_2.

		Vessel X	Vessel Y	Vessel Z
	Vessel O Ringer thermo-barometric control $V_F = 3$ cc.	$V_F = 3·5$ cc. $V_G = 4·08$ cc. $K_{O_2} = 0·365$ $K_{CO_2} = 0·554$	$V_F = 1·0$ cc. $V_G = 6·28$ cc. $k_{O_2} = 0·554$ $k_{CO_2} = 0·608$	For bicarbonate estimation at t° $V_F = 1·0$ cc. $V_G = 5·85$ cc.
Time	Dry wt. of tissues	3·85 mg.	3·73 mg.	5·18 mg.
t°	Shake 15′ to attain equilibrium	—	—	Section removed
$t^\circ + 60'$	Shake for 60′	Increase of pressure $H = +43$ mm.	Increase of pressure $h = +57$ mm. Section removed	—
	Manometric estimation of bicarbonate concentrations	—	$B_2 = 487$ mm.^{3}	$B_1 = 518$ mm.^{3}

From the increases of pressure observed in the manometers attached to the vessels X and Y, the gas exchange is calculated in the usual way.

x_{O_2} = mm.^{3} O_2 consumed in vessel Y in 60′ = 27·6 mm.^{3}

$x^{O_2}_{CO_2}$ = mm.^{3} CO_2 evolved (from glycolysis and respiration) in vessel Y in 60′ = 64·6 mm.^{3}

The bicarbonate estimations are carried out with aliquot portions of the Ringer solutions from vessels Y and Z, together with a concurrent estimation of the bicarbonate concentration of the standard Ringer solution. The manometric method used is described by Warburg [1926]. As a check on the value obtained for vessel Y, the bicarbonate concentration in vessel X may similarly be determined. This was not carried out as a routine procedure since several such preliminary estimations for checking purposes showed it to be superfluous.

Expressing the bicarbonate concentrations as mm.^{3} CO_2 per cc. solution,

$B_0 = C_{NaHCO_3}$ of standard Ringer solution $= 558$ mm.^{3}

$B_1 = C_{NaHCO_3}$ at $t° = 518$ mm.^{3} (vessel Z).

$B_2 = C_{NaHCO_3}$ at $t° + 60' = 487$ mm.^{3} (vessel Y).

Therefore

$$\text{respiratory quotient} = \frac{x_{CO_2}^{O_2} - \left[B_0 - \frac{3\cdot73}{5\cdot18}(B_0 - B_1) - B_2\right]}{x_{O_2}} = 0\cdot812.$$

Table II gives a series of the results obtained, with the simultaneously determined metabolism quotients.

Table II. *Simultaneous measurements of the carbohydrate metabolism and the respiratory quotient.*

Tumour	A Respiration Q_{O_2}	B Aerobic glycolysis assuming R.Q. is unity $Q_M^{O_2}$ (1)	C Aerobic glycolysis based on R.Q. found $Q_M^{O_2}$ (2)	D Anaerobic glycolysis $Q_M^{N_2}$	E Respiratory quotient
Tar carcinoma 173	−12·3	+10·8	+14·1	+33·4	0·735
,,	−10·2	+12·0	+14·2	+23·4	0·790
,,	−7·4	+9·9	+11·3	+15·3	0·812
Tar carcinoma 2146	−19·2	+13·9	+9·7	+26·3	1·220
,,	−14·8	+11·7	+12·8	+20·6	0·772
,,	−15·2	+13·5	+14·7	+27·4	0·923
Crocker sarcoma	−11·7	+10·2	+12·1	+24·0	0·839
,,	−8·8	+6·8	+8·5	+16·3	0·805
,,	−13·2	+10·6	+15·2	+22·8	0·649
Jensen's rat sarcoma	−5·9	+9·4	+11·0	+21·6	0·722
,,	−12·2	+11·1	+13·6	+21·3	0·794
,,	−12·8	+14·5	+19·1	+26·5	0·638

The aerobic glycolysis has hitherto been derived by deducting the measured respiration from the measured total CO_2 evolved through the operation of the combined respiratory and glycolytic processes. The assumption has been made by Warburg and other workers with these methods that the respiratory quotient is unity, under the conditions of the manometric experiment. In column B the aerobic glycolysis is given, as reckoned on this basis.

In column C the aerobic glycolysis is calculated from the value of the respiratory quotient experimentally obtained. Since the respiratory quotients, with one exception, are less than unity, the aerobic glycolysis thus found is higher than that calculated on Warburg's assumption. The effectiveness of the respiration in checking glycolysis is consequently less pronounced than is indicated by the Meyerhof quotients in Table I.

Effect of glycolysis on respiration.

In connection with an investigation into the possibility of tumour tissue metabolising pentoses, results were obtained which suggest that the glycolytic activity of tumours may act as a partial check on their respiratory powers.

The metabolism values included in Tables III and IV show that tumour tissue does not utilise xylose. The magnitudes of the respiration, whether measured in media free from sugar or with xylose added, are almost identical. No splitting of xylose occurs, with formation of acidic products, either aerobically or anaerobically.

(*a*) *Respiration measurements.* These were carried out in Ringer solution containing 0·0025 mol. of sodium bicarbonate per litre. The initial p_H of the solution was approximately 8·2. Concentrated potash was used as absorbent for CO_2. Two series of tissues were used in these measurements; eleven transplantable animal tumours, and rat liver and kidney, representative of normal tissues.

Table III *a* summarises the findings with tumour tissues.

Table III *a*. *Respiration of tumour tissues in Ringer solution, with and without sugar additions.*

C_{NaHCO_3} = 0·0025 mol. per litre. Gas phase O_2.

Tissue	Q_{O_2} Glucose added 0·2 %	Q_{O_2} Xylose added 0·2 %	Q_{O_2} No sugar added
Tar carcinoma 2146	−10·7	−12·1	−12·6
,,	−13·1	−16·6	−16·9
Jensen's rat sarcoma	−14·3	−16·6	−16·9
,,	−11·1	−13·9	−12·5
,,	−9·8	−11·0	−11·1
,,	−10·4	−13·3	−13·1
,,	−12·1	−14·0	−14·3
,,	−7·2	−9·0	−9·2
,,	−14·1	−16·7	−16·9
,,	−10·8	−13·1	−12·7
,,	−9·7	−11·0	−11·2
Average	−11·2	−13·4	−13·4

With glucose-containing Ringer solution, the values obtained were consistently somewhat lower than those obtained in the absence of glucose.

The average lowering in the examples recorded was about 10 %.

Table III *b* shows the results obtained with normal tissues. Though the measurements were made under identical conditions, the effect of the addition of glucose was not to lower the respiration but, on the average, to raise it slightly.

Table III *b*. *Respiration of normal tissues, with and without sugar additions.*

C_{NaHCO_3} = 0·0025 mol. per litre. Gas phase O_2.

Tissue	Q_{O_2} Glucose added 0·2 %	Q_{O_2} Xylose added 0·2 %	Q_{O_2} No sugar added
Mouse liver	−8·8	−8·2	−6·6
Rat liver	−10·5	−9·8	−11·1
,,	−7·3	−7·6	−6·4
,,	−11·7	−12·0	−12·1
,,	−9·2	−8·9	−8·9
,,	−6·1	−5·2	−5·5
Rat kidney	−21·6	−20·3	−20·0
,,	−16·6	−15·0	−15·0
,,	−16·6	−17·6	−14·9
,,	−22·6	−22·2	−21·4
Average	−13·1	−12·7	−12·2

Since the arbitrary conditions under which these measurements of respiration were made deviate considerably from normal physiological conditions, particularly with respect to the p_H and the bicarbonate concentration, their general significance is doubtful. The implied checking of respiration by glycolytic activity was tested under more appropriate conditions, which are recorded in the following section.

(*b*) *General metabolism measurements.* The standard solutions for the simultaneous determination of respiration and aerobic glycolysis were employed, with $C_{NaHCO_3} = 0{\cdot}025$ mol. per litre and $p_H = 7{\cdot}35$.

Table IV gives a summary of the results obtained.

Table IV. *Comparison of tumour metabolism in presence of glucose and xylose.*

$C_{NaHCO_3} = 0{\cdot}025$ mol. per litre. Gas phase = 5 % CO_2 in O_2.

	Glucose added 0·2 %		Xylose added 0·2 %		
Tissue	Q_{O_2}	$Q_M^{O_2}$	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Crocker sarcoma	- 8·3	+ 12·1	- 10·8	–	+ 1·2
"	- 10·6	+ 14·5	- 11·4	–	+ 1·1
"	- 13·6	+ 15·5	- 14·5	–	+ 1·6
"	- 9·9	+ 16·8	- 12·1	–	+ 0·8
"	- 15·8	+ 17·8	- 15·8	–	+ 1·0
Jensen's rat sarcoma	- 11·0	+ 9·3	- 13·1	–	+ 1·3
"	- 11·6	+ 12·1	- 13·7	–	+ 0·9
"	- 9·8	+ 8·9	- 11·2	–	+ 1·2
Average	- 11·3	+ 13·4	- 12·8	–	+ 1·1

Tissues from the same animal were used in concurrent estimations in the presence of glucose and of xylose. The results show an appreciable lowering of the respiration, about 12 % on the average, in the glucose-containing Ringer solution when compared with the xylose-containing Ringer solution. With xylose present no acid formation results through sugar splitting, either aerobically or anaerobically.

The tentative conclusion is that glycolytic activity exerts a significant checking effect on the capacity for respiration of tumour tissue.

Influence of environment on the carbohydrate metabolism of Jensen's rat sarcoma.

A few observations of the carbohydrate metabolism of Jensen's rat sarcoma were made with the intention of confirming the numerous consistent values found by Warburg and his collaborators.

Anomalous results were obtained, a tendency for the respiration to be high with respect to the anaerobic glycolysis being often noticed. The value of *U* was often negative. In Table V are collected the results of a few of these preliminary determinations.

The tumour strains of the Imperial Cancer Research Fund are propagated by subcutaneous grafts in the flank.

Table V. *Jensen rat sarcoma.*

Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Meyerhof quotient	U
−15·2	+13·5	+26·2	0·8	−4·2
−11·7	+10·2	+24·0	1·2	+0·6
−13·2	+10·6	+20·1	0·7	−6·3
−12·2	+11·1	+18·8	0·6	−5·6
−12·8	+14·5	+26·5	1·0	+0·9
−10·9	+ 9·3	+21·6	1·1	−0·2

The work of Campbell [1926] on tissue oxygen and carbon dioxide tensions has shown, by the method of gas injection, that the tensions of these gases vary in different parts of the body. Campbell and Cramer [1928] showed that rapidly growing implanted tumours in rats and mice show a greatly diminished rate of growth during prolonged exposure to low oxygen pressures. These observations suggested the possibility that the abnormal results recorded in Table V might be due to effects determined by the site of transplantation. Accordingly comparative series of metabolism measurements were made on two series of tumours, one series transplanted subcutaneously and the other intraperitoneally. The tumour used was Jensen's rat sarcoma, the rats chosen being of about the same age, 2 to 3 months. The inoculations were made with material from the same tumour and at the same date.

The tumours grown intraperitoneally are relatively more haemorrhagic than those grown subcutaneously and develop as a number of nodules adherent to neighbouring surfaces. As growth proceeds, these nodules unite, forming a large coherent mass. Isolated nodules provide the best material for metabolism experiments; microscopically they show numerous mitotic figures and no necrotic areas are visible.

The results are collected in Table VI.

Table VI. *Comparison of metabolism of Jensen rat sarcoma when transplanted subcutaneously and intraperitoneally.*

	Subcutaneous transplantations				Intraperitoneal transplantations			
	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	U	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	U
	−19·3	+12·3	+28·4	−10·2	− 6·2	+12·6	+31·6	+19·2
	−16·1	+19·6	+32·6	+ 0·4	− 7·6	+11·9	+29·0	+13·8
	−16·4	+16·8	+32·8	0	−12·3	+15·1	+26·9	+ 2·3
	−13·6	+13·7	+27·6	+ 0·4	−11·1	+15·4	+34·0	+11·8
	−18·9	+22·5	+31·6	− 6·2	− 8·6	+14·2	+25·4	+ 8·2
	−19·3	+20·1	+33·4	− 5·2	−11·1	+15·0	+25·4	+ 3·2
	−15·2	+14·1	+27·3	− 3·1	−13·3	+22·3	+37·5	+10·9
	−16·6	+15·5	+27·3	− 5·9	−10·3	+22·2	+37·5	+16·9
	−19·1	+25·8	+36·3	− 1·9	−12·3	+ 8·6	+25·0	+ 0·4
	−27·9	+26·5	+36·4	−19·4	− 9·6	+ 8·4	+24·7	+ 5·5
	−18·5	+24·5	+34·8	− 2·2	−12·4	+17·9	+33·6	+ 8·8
	−14·2	+24·2	+34·9	+ 6·5	−12·9	+17·1	+32·8	+ 7·0
	−15·3	+18·0	+30·6	0	−15·7	+17·2	+34·9	+ 3·5
	−12·0	+17·5	+34·5	+10·5	−15·9	+17·2	+34·9	+ 3·1
	−16·9	+23·3	+34·9	+ 1·1	−12·6	+17·4	+37·3	+12·1
	−16·7	+25·4	+30·9	− 2·5	− 7·0	+18·4	+35·3	+21·3
	−18·1	+25·2	+33·5	− 2·7	—	—	—	—
Av.	−17·3	+20·3	+32·2	− 2·4	−11·2	+15·7	+31·6	+ 9·2

A consideration of these results leads to the following conclusions.

1. All the tumours grafted intraperitoneally show a carbohydrate metabolism conforming to that found by Warburg. A positive U, or excess fermentation, is a common property.

2. A large majority of the tumours grafted subcutaneously show a value for U which is either negative or near zero.

3. The magnitude of the respiration of the tumours grafted subcutaneously is considerably higher than that of the tumours grafted intraperitoneally. This increased respiration is in the neighbourhood of 50 %.

A corresponding increase in the aerobic glycolysis amounts to about 30 %.

4. The anaerobic glycolysis is, on the average, of the same magnitude in each series.

Summary and discussion.

1. Estimations of the carbohydrate metabolism of several strains of mouse tumours are recorded. Great deviations from the standard values found for tumours of rat, fowl and a limited series of human tumours were observed in many cases.

Wide variations are shown to occur between tumours of different strains, and also between members of the same strain.

The most noticeable feature is the number of cases of high respiration, both in its absolute value and also in its relation to the aerobic and anaerobic glycolysis. This respiration is ineffective in checking the aerobic glycolysis, its activity in this direction being, in some cases, less than 10 % of that found in the case of working muscle, and in many mammalian tumours.

Some factors which might operate in causing these variations are changes in the respiratory quotient, differences of environment during growth, efficiency of blood supply, and the generally higher metabolic rate of the mouse as compared with larger animals.

2. A manometric method for the simultaneous measurement of the carbohydrate metabolism and the respiratory quotient is briefly described, based on the fact that the glycolysis effected by tumour tissue is a pure lactic fermentation.

The respiratory quotients with one exception were found to be below unity. This would tend to make the actual aerobic glycolysis relatively higher than that usually recorded, since the assumption has hitherto been made that a respiratory quotient of unity would result from the experimental conditions.

The results again illustrate the ineffectiveness of respiration in checking glycolysis.

3. Xylose is not metabolised by tumour tissue.

4. Evidence is brought forward which suggests that the glycolytic activity of tumours exerts a checking effect on their respiration.

5. The carbohydrate metabolism of tumours is to some extent influenced by the environment in which they grow. This is demonstrated by the study of two series of Jensen's rat sarcomata, simultaneously transplanted, one series subcutaneously and the other intraperitoneally.

The respiration of the subcutaneous growths was, on the average, 50 % higher than that of the intraperitoneal growths. The majority of these subcutaneous tumours do not exhibit a positive value for the excess fermentation, which was, until recently, regarded by Warburg as a criterion for the metabolism of tumour tissue.

The correlation of these differences with the normal tissue tensions of CO_2 and O_2 is difficult. Campbell found the oxygen tension in the abdominal cavity 50 % higher than under the skin, the CO_2 tensions being approximately the same.

The higher respiration found in these two series of tumours corresponds to the lower O_2 tension in the surrounding tissues, and *vice versa*. It is obvious that other factors which have not yet been analysed are operative.

The general result of these observations is to emphasise the difficulty of including the wide variations found in the carbohydrate metabolism of tumour tissue in one generalisation.

The constant factor is the possession of a high aerobic glycolysis, which, though not specific for tumour tissue, is a source of energy available for uncontrolled proliferation.

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LXII. EXPERIMENTS ON NUTRITION. IX.

COMPARATIVE VITAMIN B VALUES OF FOODSTUFFS. PULSES AND NUTS.

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THE good value of the "katjang-idjo" bean (*Phaseolus radiatus*) as a preventive and curative of beriberi was described by Grijns [1901]. Cooper [1912] and Chick and Hume [1917] subsequently made some quantitative tests with lentils and dried peas. The comparative values of these two pulses, with wheat germ as 100, was given in the Report on accessory food factors of the Medical Research Council (1924) as 80 and 40. Cajori [1920] found that the addition of 1 g. of various nuts to the diet restored growth to rats declining in weight. Observations by McCollum, Simmonds and Pitz [1917] on the nutritive value of the white bean led to the statement that 25 % of the bean in the diet of rats furnished sufficient vitamin B. Similar experiments with the soya bean by Daniels and Nichols [1917] and with the peanut by Daniels and Loughlin [1918] showed that 50 and 56 % of these foods supplied enough vitamin B for the rat. Also, Johns, Finks and Paul [1919] found that 75 % of coconut press cake had enough vitamin B for the rat.

In view of the scanty information about these foodstuffs in regard to their vitamin B content, and in order to obtain their comparative values more closely, we have undertaken this series of experiments. Our results must at present be considered as the vitamin B_1 + vitamin B_2 value, though the symptom of polyneuritis has been taken as far as possible as the criterion of the amount of vitamin in the foods. As our method of testing foodstuffs for vitamin B is very laborious the work has been limited to the more common varieties of pulses and nuts.

EXPERIMENTAL.

As in our former testing of foodstuffs for vitamin B [Plimmer, Rosedale, Raymond and Lowndes, 1927] pigeons have been used. They have been kept in pairs in suitably sized cages on the roof of the building. The earlier experiments were made with the foodstuff mixed with white rice and fish meal and with rearing as the standard of the vitamin B value. As the series of tests extended it was noticed that the birds scattered and separated the mixture

and so white flour was used in place of white rice. The ingredients were mixed into a dough and the dough divided into pills which were dried in the air, or at 37°. This mixture could not be separated, and thus the birds had to eat the mixture required for experimentation. The standard of rearing was considered too high and the standard of maintenance for at least 26 weeks was substituted. It had often been found that a period of 15 weeks was not long enough, and occasionally birds were observed to suffer from polyneuritis after as long a time as 30 weeks.

The result with each foodstuff is given shortly together with the times of the experiment and the weights, etc., in the tables.

Split peas.

In the first trial the birds were on the split pea diet for 106 weeks and showed no signs of any ill-effect. The other tests showed that for maintenance the diet must contain 30 % split peas. Rearing was possible on 40 %, but precarious, and not improved on raising to 50 %. Table I gives the details.

Table I. *Split peas.*

		Diet			Weights, g.			
Date	Time in weeks	Split peas	White rice	Fish meal	Cock No. 25/15	Hen No. 25/10	Number of eggs	Result
17. vi. 25		40	55	5	410	350	22 in Sept., Nov., Feb., Mar., Apr., May, June, July, Sept.	First 2 hatched and reared; 2 unfertile; 4 deserted; 9 fertile and deserted; 5 hatched and died in 2nd week
to								
12. x. 26	69				495	410		
13. x. 26		50	45	5			2 in Nov.	Both hatched and died in 1st week
to								
4. i. 27	12				515	435		
			White flour					
5. i. 27		40	55	5			10 in Feb., Mar., Apr., May	All fertile and deserted
to								
31. v. 27	21				480	390		
1. vi. 27		50	45	5			2 in June	Deserted
to								
28. vi. 27	4				485	430		
					No. 26/28	No. 26/30		
29. i. 27		30	65	5	415	505	16 in Mar., Apr., May, June, July	All were broken and deserted, some were fertile
to								
22. vii. 27	25				420	430		
					No. 21	No. 25/34		
20. i. 27		20	75	5	480	500	None	Both birds showed paralysis, but only hen seen with retracted head. Died
to								
13. ii. 27	4				310	315		
					No. 9	No. 10		
16. xii. 26		10	85	5	465	465	None	Both paralysed. Cock but not hen seen with retracted head. Died
to								
17. i. 27	5				390	290		

Whole dried green peas.

The first test was started at the same time as that with split peas. The pair were on the diet for 87 weeks altogether and on changing to white flour for another 15 weeks. The pair of birds, previously on 30 % split peas, after a period on brown rice, were given a diet with 30 % green peas to test for maintenance. They remained for 27 weeks in good condition. There seemed thus no difference between whole green peas and split peas and no further trial of green peas was then made. The tests with green peas at 20 % and 10 % were made later (Table II), and showed that 30 % dried green peas was the minimum for maintenance and that the husk contained no greater concentration of vitamin B than the endosperm.

Table II. *Whole dried green peas.*

Date	Time in weeks	Diet: Green peas	Diet: White rice	Diet: Fish meal	Weights, g.: Cock No. 25/1	Weights, g.: Hen No. 25/13	Number of eggs	Result
17. vi. 25 to 1. vi. 26	50	40	55	5	365	315	14 in Sept., Oct., Dec., Feb., Apr., May	6 hatched and reared; 4 fertile and deserted; 4 unfertile
2. vi. 26 to 28. ix. 26	17	30	65	5	410	350	15 in June, July, Aug., Sept.	All deserted; some fertile
29. ix. 26 to 15. ii. 27	20	40	55	5	450	360	4 in Oct.	All deserted
			White flour					
16. ii. 27 to 31. v. 27	15	40	55	5	490	400	12 in Feb., Mar., Apr., May	All deserted
1. vi. 27 to		50	45	5	390	350	4 in June	All deserted
28. vi. 27	4				420	360		
					No. 26/28	No. 26/30		
14. ix. 27 to		30	65	5	410	435	10 in Oct., Feb., Mar.	2 hatched and died in 1st week; 8 deserted
21. iii. 28	27				450	485		
					No. 96	No. 65		
10. x. 28 to		20	75	5	395	405	None	
9. i. 29 to	13				375	385		
3. iv. 29 to	25				340	310		Hen died with large heart. Cock showed retracted head, and died
24. iv. 29	28				300			
					No. 77	No. 128		
30. i. 29 to		10	85	5	495	445		
13. iii. 29	6				320	320	None	Both showed paralysis, the cock with retracted head. Both died in 7th week

Lentils.

Altogether the pair of birds were kept 79 weeks on lentils and rice. There was an improvement in rearing on raising to 50 % lentils.

The trial with 20 % lentils was made on the birds previously used with split peas. They were given brown rice in the interval.

The details given in Table III show that for maintenance 30 % lentils are required. Rearing was scarcely possible on 40 %, but fairly good on 50 %.

Table III. *Lentils.*

Date	Time in weeks	Diet: Lentils	Diet: White rice	Diet: Fish meal	Weights, g.: Cock No. 33	Weights, g.: Hen No. 25/5	Number of eggs	Result
5. viii. 25 to 29. vi. 26	47	40	55	5	420	355	9 in Nov., Mar., Apr., May	2 unfertile; 3 hatched and died; 1 reared but abnormal; 1 dead at hatch; 2 fertile, deserted
30. vi. 26 to 8. ii. 27	32	50	45	5	420	440	7 in July, Aug., Sept., Oct.	2 hatched and died; 2 hatched and reared; 3 fertile, deserted
			White flour					
9. ii. 27 to 1. vi. 27	17	40	55	5	450 420	510 440	5 in Mar., Apr., May	All deserted. Birds escaped
					No. 75	No. 70		
9. ii. 27 to 22. vi. 27	19	30	65	5	430 380	435 425	6 in May, June, July	All deserted. Hen escaped
						No. 54		
6. vii. 27 to 25. viii. 27	7				395 410	340 370	2 in July	Deserted
					No. 25/15	No. 25/10		
18. viii. 27 to 12. x. 27	8	20	75	5	475 395	395 285	2 in Aug.	Deserted. Both birds showed retracted head and were cured

Haricot beans.

The first pair of birds did not like the beans and only ate them if compelled by not getting a new supply of food until the old had been cleared up.

The tests as given in Table IV showed that 40 % was required for maintenance.

The same pair, after a period of about 5 weeks on wheat, were started on 30 % haricot beans. At death both birds were found to have tumours.

Table IV. *Haricot beans.*

Date	Time in weeks	Diet: Haricot beans	Diet: White rice	Diet: Fish meal	Weights, g.: Cock No. 25/14	Weights, g.: Hen No. 25/19	Number of eggs	Result
1. vii. 25		40	55	5	360	345		
to								
13. x. 25	15				230	315	None	Cock ill, chloroformed
to								
23. xii. 25	25					275		Hen ill, chloroformed
			White flour		No. 5	No. 4		
2. iii. 27		40	55	5	435	375	18 in Mar., Apr., May, June, July	Generally deserted after a few days, some found fertile
to								
10. viii. 27	23				440	395		
					No. 84	No. 37		
24. xi. 27		20	75	5	450	400		
to								
4. i. 28	6				320	320	None	Cock died with very wet tissues
to								
4. iv. 28	20					325		Hen died with typical polyneuritis
					No. 5	No. 4		
15. ix. 27		30	65	5	425	425	6 in Sept., Oct.	Deserted
to								
9. i. 28	17				475	340		Hen died with growth in oviduct
						No. 69		
29. ii. 28	24				445	305	9 in Mar., Apr., May, June	All deserted
to								
18. v. 28	40				305	340		Cock died; tumour in testis
to								
12. ix. 28	28					295		Hen showed signs of polyneuritis

Table V. *Soya beans.*

Date	Time in weeks	Diet: Soya beans	Diet: White flour	Diet: Fish meal	Weights, g.: Cock No. 52	Weights, g.: Hen No. 42	Number of eggs	Result
8. ii. 28		30	65	5	490	530	10 in Apr., May, June, July, Aug.	All unfertile
to								
22. viii. 28	28				425	500		
					No. 100	No. 233		
24. iv. 28		40	55	5	410	425	4 in May, June	1 fertile; 3 unfertile
to								
27. vi. 28	9				415	475		
28. vi. 28		20	75	5			2 in July	Hatched and died
to								
7. xi. 28	19				345	370		Hen slightly paralysed and died; cock better
to								
20. i. 29	31				420			
					No. 37	No. 332		
17. x. 28		10	85	5	445	385	None	Both showed paralysis in 5th week; hen with retracted head
to								
14. xi. 28	4				315	315		

Soya beans.

The details in Table V tend to indicate that 20 % soya bean suffices for maintenance. On this quantity the birds began to lose weight, but slowly regained it. The hen in the 18th week suffered from an eye infection and died. Both birds ate very little during the time of the experiment and never looked really well. We have several times noticed that pigeons which eat little can maintain themselves on a diet with just too little vitamin B for a long time but not indefinitely. It cannot be considered that 20 % soya bean is enough; 30 % is nearer the minimum quantity.

Peanuts.

The test of peanuts is shown in Table VI.

20 % is enough for maintenance; rearing was possible on 40 %. Similar figures, twice as much vitamin B being required for rearing as is needed for maintenance, have been found in previous experiments.

Table VI. *Peanuts.*

Date	Time in weeks	Diet: Peanuts	Diet: White rice	Diet: Fish meal	Weights, g.: Cock No. 25/26	Weights, g.: Hen No. 43	Number of eggs	Result
26. ii. 26		40	55	5	455	430	8 in Mar., May, July, Aug.	5 hatched and reared; 1 hatched and died; 1 addled; 1 unfertile
to								
29. ix. 26	31				430	415		
30. ix. 26		30	65	5			1 in Oct.	Fertile, deserted
to								
9. ii. 27	19				490	485		
			White flour					
10. ii. 27		30	65	5				
to						No. 23		Hen escaped
9. iii. 27	4				465	450	4 in Apr., May	New hen.
to								Fertile, deserted
25. v. 27	15				360	480		Cock not well; experiment stopped
					No. 83	No. 25/27		
23. ii. 27		20	75	5	410	385	16 in Feb., Mar., Apr., June, July, Aug.	3 hatched and died; 1 hatched and reared; 12 fertile, deserted
to								
24. viii. 27	26				390	350		
					No. 83	No. 25/27		
6. x. 27		30	65	5	415	390	10 in Oct., Nov., Dec., Jan., Feb.	2 hatched and died; 7 fertile; 1 unfertile, deserted. Hen suddenly not well; experiment stopped
to								
14. iii. 28	23				435	280		
					No. 88	No. 25/5		
16. vi. 27		10	85	5	395	405	2 in July	Unfertile, deserted
to								
31. viii. 27	11				360	325		Hen had polyneuritis and died
to								
2. xi. 27	20				325			Cock had polyneuritis and was cured with 1 g. of wheat germ ("bemax")

Almonds.

The vitamin B value of almonds was tested both with ground almonds, such as are used in making cakes, and with whole almonds, which were put through a mincer before being incorporated with white flour and fish meal. The two varieties were used to ascertain if the skin contained any vitamin B (see Table VII).

The first pair of birds were on the ground almond diet for 72 weeks in all. The other pairs on smaller amounts of ground almonds indicated that 40 % was required for maintenance.

The experiments with whole almonds (Table VIII) were started with 20 % and 30 % in the diet, the one with 40 % after the other results were known.

The minimum quantity of whole almonds for maintenance was thus 40 %, the same as with ground almonds. The skins do not appear to contain more vitamin B than the rest of the seed.

Table VII. *Ground almonds.*

Date	Time in weeks	Diet: Ground almonds	Diet: White rice	Diet: Fish meal	Weights, g.: Cock No. 1	Weights, g.: Hen No. 5209	Number of eggs	Result
1. iv. 26 to		40	55	5	485	420	8 in Apr., May, June	2 unfertile; 6 fertile; no hatching
14. vii. 26	15				445	400		
15. vii. 26 to		50	45	5			8 in July, Aug., Oct., Mar.	All fertile; 1 hatched and died
9. iii. 27	34				490	480		
			White flour					
10. iii. 27 to		50	45	5			6 in Apr., May	All deserted; some fertile
25. v. 27	11				470	410		
26. v. 27 to		60	35	5			8 in June, July, Aug.	All fertile, deserted
24. viii. 27	12				470	440		
					No. 89	No. 49		
9. xi. 27 to		40	55	5	385	370	14 in Dec., Jan., Feb., Mar., Apr., May	Mostly fertile, no hatching
14. iii. 28 to	18				400	365		Cock had head retraction in 17th week and was cured by 1 g. of dried yeast and remained well
20. vi. 28	32				380	350		
					No. 76	No. 449		
1. ix. 27 to		30	65	5	435	485		Cock had head retraction in 9th week and died; hen ill, but recovered
26. x. 27 to	8				425	410		
8. ii. 28	19					430		
					No. 63	No. 92		
9. ii. 28 to		30	65	5	440	420		Both birds had head retraction and died
4. iv. 28	8				280	275		
					No. 76	No. 16		
23. vi. 27 to		20	75	5	420	325		Both birds showed head retraction and were cured
20. vii. 27	4				290	260		

Table VIII. *Whole almonds.*

Date	Time in weeks	Diet: Whole almonds	Diet: White flour	Diet: Fish meal	Weights, g.: Cock No. 67	Weights, g.: Hen No. 128	Number of eggs	Result
15. iii. 28 to		30	65	5	380	425	2 in Mar.	Both hatched and died in 1st week with large hearts and wet tissues
23. v. 28	10				300	380		Cock died 25. iii. 28 with paralysis, but head not seen retracted. Hen had head retracted on 29. v. 28 and was cured with 0·75 g. dried yeast
					No. 83	No. 294		
25. iv. 28 to		20	75	5	415	415	2 in Apr.	Deserted
23. v. 28	5				355	285		Cock had head retracted in 5th week and was cured with 0·5 g. dried yeast. Hen had head retracted in 6th week and was cured with 0·5 g. wheat germ
					No. 2569	No. 50		
17. v. 28 to		40	55	5	345	470	2 in May	1 unfertile; 1 fertile, deserted
31. x. 28 to	24				375	435		Cock escaped
14. xi. 28	26					465		

Table IX. *Hazel nuts.*

Date	Time in weeks	Diet: Hazel nuts	Diet: White flour	Diet: Fish meal	Weights, g.: Cock No. 57	Weights, g.: Hen No. 6	Number of eggs	Result
7. xii. 27 to		30	65	5	520	475	6 in Mar., Apr., May	All fertile; 5 hatched and died
13. vi. 28	27				475	395		
					No. 96	No. 65		
28. i. 27 to		20	75	5	380	425	8 in Mar., Apr., May, June	2 unfertile; 6 fertile, all deserted
8. viii. 28 to	32				350	275		
22. viii. 28	34				330	365		Hen showed polyneuritis; cured with 1 g. wheat germ
					No. 57	No. 6		
13. vi. 28 to		10	85	5	475	395		
4. vii. 28	3				350	300		Both had polyneuritis and both cured with 0·5 g. marmite

Hazel nuts.

The test of hazel nuts is shown in Table IX. The pair on 30 % after a period of 27 weeks were then given 10 %. The minimum quantity for maintenance of

adult pigeons thus seemed to be 20 %. It may be noted that the hen became ill after 30 weeks and showed polyneuritis. This was possibly the result of laying so many eggs.

Chestnut.

For the purpose of these experiments dried chestnuts were used, as they could be obtained during almost the whole year, and as they were in this state more easily comparable with almonds and hazel nuts.

As a preliminary to the trial a pair of birds were given the dried chestnut with 5 % fish meal to see if they would eat it. It was eaten well and the birds increased in weight. In the first trial the birds were on the chestnut diet for 75 weeks. They were then given a diet of brown rice with 5 % fish meal and later tried with 30 % chestnut. Table X shows that the quantity of dried chestnut for maintenance is 40 %. Some rearing was possible on this amount, but it was better with 50 %.

Table X. *Chestnut.*

		Diet			Weights, g.			
Date	Time in weeks	Chestnut	White rice	Fish meal	Cock No. 7	Hen No. 8	Number of eggs	Result
10. ii. 26		95	0	5	500	385		
to								
3. iii. 26	3				555	445		
4. iii. 26		50	45	5			4 in Mar., Apr.	All hatched and reared
to								
9. vi. 26	14				465	380		
10. vi. 26		40	55	5			14 in June, July, Aug., Sept., Feb.	2 hatched and reared; 3 unfertile; 9 fertile, deserted
to								
16. iii. 27	40				505	425		
			White flour					
17. iii. 27		40	55	5			10 in Mar., Apr., May	All deserted
to								
1. vi. 27	11				445	405		
2. vi. 27		50	45	5			5 in June, July	All deserted
to								
10. viii. 27	10				455	385		
			Brown rice					
11. viii. 27	8	0	95	5	510	430		
to								
5. x. 27								
			White flour		No. 14	No. 13		
24. viii. 27		20	75	5	450	395	2 in Aug.	Deserted
to								
20. ix. 27	4				335	285		Cock showed retracted head. Cured with 1 g. wheat germ
to								
26. x. 27	10					260		Hen had paralysis and died
					No. 7	No. 8		
6. x. 27		30	65	5			2 in Oct.	Deserted
to								
23. xi. 27	7				420	340		Cock showed retracted head. Cured with 1 g. of wheat germ
to								
30. xi. 27	8				420	330		Hen showed retracted head. Cured with 1 g. of wheat germ

Coconut.

The coconut used in these experiments was the material commonly employed for making cakes and sweets, and consisted of the dried and flaked flesh of the nut.

It was expected that this food would show about the same value as the other nuts, and the first trial was made with 40 % mixed with white rice. The birds did not appear to eat the coconut, and after 6 weeks the trial was stopped as the birds had lost weight and looked ill. They were put upon millet, their former diet, for the next 3 weeks and were then given coconut mixed with 5 % fish meal. The food was not liked and little was eaten. Both birds showed paralysis and died.

Table XI. *Coconut.*

Date	Time in weeks	Diet: Coco-nut	Diet: White rice	Diet: Fish meal	Weights, g.: Cock No. 15	Weights, g.: Hen No. 16	Number of eggs	Result
27. i. 26		40	55	5	495	430		
to								
10. iii. 26	6				340	360		Ate mostly rice
31. iii. 26		95	0	5	405	425		
to								
28. iv. 26	4				335	355		Cock showed retracted head and died
to								
23. vi. 26	12					280		Hen showed paralysis and died
					No. 25/14	No. 25/27		
2. vi. 26		95	0	5	365	345		
to								
7. vii. 26	5				290	285		Cock showed retracted head and died
to								
21. vii. 26	7					245		Hen showed retracted head. Cured with 1 g. yeast

Table XII. *Coffee.*

Date	Time in weeks	Diet: Green coffee	Diet: White rice	Diet: Fish meal	Weights, g.: Cock No. 7	Weights, g.: Hen No. 8	Number of eggs	Result
5. i. 28		30	65	5	600	445		
to								
29. ii. 28	8				345	305		Cock showed retracted head. Cured with 1 g. wheat germ
to								
21. iii. 28	11				325	285		Cock died
to								
28. iii. 28	12					255		Hen was paralysed and died
		Roasted coffee			No. 39	No. 50		
29. xii. 27		30	65	5	380	455		
to								
1. ii. 28	5				225	305		Both birds died
					No. 84	No. 62		
10. ii. 28		50	45	5	370	385		
to								
9. iii. 28	4				245	255		Both birds died

As coconut is such a common foodstuff one more trial was made. The former result was confirmed (see Table XI).

The flesh of the coconut, as prepared for making cakes, thus appeared to contain no vitamin B.

Coffee.

It was not expected that roasted coffee would contain vitamin B, but it was felt necessary to try the experiment. Two experiments were made with 30 and 50 % coffee. The birds died after the 5th and 4th weeks respectively.

At the same time a trial was made with 30 % green coffee. As the birds lived for 8 to 12 weeks it would appear that green coffee contained some vitamin B. A higher proportion in the diet was not tried. The vitamin in the seed is thus destroyed during the roasting (see Table XII).

Discussion and summary.

Previous work upon the vitamin B value of pulses and nuts suggested that the pulses had varying values, but that nuts were of more uniform value. Our results do not support the older observations. Pulses have been found to have a very similar vitamin B value, whilst nuts showed considerable variation. Hazel nuts were of highest value, coconut was of no value.

It is convenient to adopt a standard for comparison. In our former work [1927] dried yeast was taken as standard. 4 % of this food was found sufficient to maintain a pair of pigeons for 26 weeks or longer. It was placed at 100. Using the same standard the pulses and nuts now tested come out as follows.

	Percentage amount in diet for maintenance	Relative vitamin B value
Dried yeast	4	100
Split peas	30	13
Whole dried green peas	30	13
Lentils	30	13
Haricot beans	40	10
Soya beans	30	13
Peanuts	20	20
Ground almonds	40	10
Whole almonds	40	10
Hazel nuts	20	20
Dried chestnuts	40	10
Coconut	No maintenance	0
Coffee, green	Over 30	Less than 13
Coffee, roasted	No maintenance	0

These figures do not compare strictly with those given for cereals in our former work, as the standard of hatching and rearing was then taken as criterion of the vitamin B value. The values of cereals are being re-determined on the standard of maintenance for a period of at least 26 weeks. The amount of whole wheat for maintenance has been found to be 40 %, whilst oatmeal and maize are showing 50 %, a higher value than was expected. Other tests are being made with fruits and vegetables. Potatoes and artichokes

at 80 % and parsnips and leeks at 60 % of the diet have been found to maintain pigeons for long periods. The other vegetables and fruits have no appreciable quantity of vitamin B.

In these experiments it has again been observed that for hatching and rearing of young pigeons more vitamin B is required than for maintenance. In many cases rearing has been possible on double the amount needed for maintenance.

As it is not possible at present in the case of these foodstuffs to differentiate vitamin B into B_1 and B_2 factors, the results must be considered as due to the two factors together, though the symptoms of polyneuritis have been taken as the indication of the value. Loss of weight cannot be regarded as an indication of vitamin B_2 value. As soon as the food mixture is not suitable for maintenance of the pigeons, consumption of the food gets less and less and the birds lose weight in consequence. Polyneuritis has never been observed without refusal of the birds to eat. Both Cooper, and Chick and Hume, have mentioned quantities of foodstuffs required to prevent loss of weight of pigeons, but our experience indicates that continuation of their experiments for a longer period would have led to polyneuritis.

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LXIII. FURTHER OBSERVATIONS ON THE EFFECTS OF LARGE DOSES OF IRRADIATED ERGOSTEROL.

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Recent observations on the effects produced in animals by excessive feeding with irradiated ergosterol have provided definite results which are at variance. Pfannensteil [1927], Kreitmair and Moll [1928], and Harris and Moore [1928] have shown that rats and some other animals become ill in a few days and die in a week or two when they are given some milligrams of this substance daily in addition to a normal diet; and Kreitmair and Moll [1928] and Kreitmair and Hintzelmann [1928] described an atrophic spleen, haemorrhagic enteritis, and extensive and severe arteriosclerosis as the prominent pathological features. On the other hand, Dixon and Hoyle [1928] were unable to confirm these results. They found that rats failed to gain in weight when fed with similar amounts of irradiated ergosterol, and when killed, after being fed in this way for about 40 days, showed calcium phosphate calculi in the urinary tract. Dixon and Hoyle used ergosterol which had been irradiated in oily solution, and they suggested that the medium in which irradiation is effected may be a deciding factor in determining the toxicity of the product as shown by feeding experiments with very large doses. In the experiments of Harris and Moore the ergosterol had been irradiated in alcoholic solution. The German observers did not state their method of irradiation.

In the experiments described in the present paper the work of Dixon and Hoyle has been repeated with the addition of certain quantitative estimations on the urine in an attempt to determine the immediate cause of the formation of the calculi found in such experimental animals.

Experimental.

Twenty-four half-grown albino rats each weighing about 180 g. were chosen for the experiment and kept throughout in cages of the Hopkins pattern, two rats in each cage. The urine was collected by Hopkins's method in 3-day periods, and that from three cages mixed and analysed together. In this way four groups, each consisting of six rats, were maintained, and two of these groups received the experimental diet and two were kept as controls. The

volume of urine passed by each group was measured separately. The contents of the three flasks from each group were poured into a graduated cylinder and the volume noted. In this way the average amount of urine passed per rat per day could be calculated. The urine was then poured back into one of the flasks; the graduated cylinder and the two remaining flasks were washed out with 20 cc. of distilled water and these washings were added to the main bulk of urine. The analyses were made on this diluted urine and allowance made for the dilution by calculation. In the earlier estimations the urine was acidified with 5 cc. of N HCl in order to dissolve any calcium phosphate which might have crystallised out: in the later estimations, which were made daily, no acid was added. When the estimations were not carried out immediately the urine was preserved with chloroform.

We intended to estimate the urinary calcium excretion, but we found that in using the ordinary Shohl and Pedley technique [1922] for the calcium estimations it was impossible to obtain with certainty a sufficiently clear solution after digesting the urine with ammonium persulphate. In the case of rats' urine this oxidation gives rise to an insoluble substance which has not yet been thoroughly investigated. As it was partly organic in nature, we considered that further investigation of the completeness of the oxidation of urates and allied substances in rats' urine by this method was necessary before it could be relied upon as a step in the estimation of calcium. For this reason we were compelled to abandon calcium estimations in the present experiments.

Urinary phosphate was estimated by Briggs's method [1922]. The acidified urine was shaken and allowed to stand; 10 cc. were withdrawn and the HCl previously added neutralised by the calculated amount of $N/10$ NaOH. The solution was then made up to 100 cc. and 1 cc. used for the estimation. During the last 9 days of the experiment daily estimations of urinary phosphates, chlorides, and p_H were made. Chlorides were determined by Whitehorn's modification of Vollhard's method. The hydrogen electrode was used for the p_H determinations. In all the estimations duplicates were done as far as possible, and gave satisfactory readings.

All the animals received a control synthetic diet for a preliminary period of 14 days. The synthetic diet consisted of caseinogen 20 %, potato starch 35 %, sugar 10 %, cacao butter 20 %, orange juice 5 %, marmite 5 %, salt mixture 5 %. The salt mixture was that given by Hume and Smith [1928], with the addition of 0·1 % potassium iodide and traces of sodium fluoride and manganese sulphate. All the animals received in addition to this diet 2 drops of a standardised cod-liver oil daily. The experimental diet contained 1 % of irradiated ergosterol in the cacao butter so that 10 g. of the completed diet contained 20 mg. of irradiated ergosterol. The irradiated ergosterol, kindly supplied by the British Drug Houses, was irradiated in oil as described by Dixon and Hoyle [1928]. Distilled water for drinking was supplied in ample quantity in glass bulbs. After the preliminary control period, half the animals were given the experimental diet, and the other half continued on the control

diet. The experimental period was continued for 45 days. In all cases the diets were given mixed into a stiff paste with a little distilled water in order to reduce scattering to a minimum. Each rat was allowed 15 g. of the dry diet daily, and the leavings from each cage were weighed each day. In this way the average quantity of food and irradiated ergosterol taken per rat could be accurately estimated.

In the urinary estimations there are two main sources of probable error, contamination of the urine with faeces and with food. It is impossible to prevent faecal particles adhering to the urine collectors occasionally; and it is impossible entirely to prevent scattering of food. In order to decide the extent of the errors involved in these ways, two estimations were made. In the first case four faecal masses were shaken up for 5 minutes with 100 cc. of distilled water. After sedimentation 1 cc. of the fluid was withdrawn for the estimation of phosphate. The amount of soluble phosphate present, however, was so small that no detectable blue colour was produced with the Briggs reagents. In the second place 2 g. of control diet were shaken up with 100 cc. of distilled water. The phosphate in this solution was then estimated and it was found that 5·5 mg. had passed into solution. Supposing that 50 cc. of urine are contaminated with 2 g. of diet, then approximately 11 mg. of phosphate are added; this is equivalent to an error of about 3 % and is negligible when the additional sources of error that are inseparable from such animal experiments are considered. But it is extremely improbable that so large a quantity of food as 2 g. was ever mixed with the urine.

Results.

All the control rats throughout the entire experiment and the experimental rats during the preliminary control period maintained an approximately steady food consumption. When the experimental rats began to take the diet containing the irradiated ergosterol they showed a decrease in appetite during the first week. During this time they lost weight to a corresponding degree and although subsequently they returned to their normal level of food consumption this was not accompanied by a gain in weight. During the remaining period of the experiment the average weight curve for the twelve experimental animals remained practically steady. This is shown in Fig. 1. The average amount of irradiated ergosterol taken per rat per day over the whole experimental period was 26·4 mg.

The daily output of urine and the urinary phosphate excretion for the control and experimental groups of rats are shown in Figs. 2 and 3 respectively. The figures for both control and both experimental groups have been averaged and presented together since there were no important differences between the two sub-groups. It will be seen that the urine output of the control group was constant within definite limits throughout the whole experiment. The daily urinary phosphate excretion varied, however, considerably from day to day and is throughout placed at a very high level relative to that of other animals.

During their control period the experimental animals were strictly comparable with the controls both as regards their urine output and urinary phosphate excretion. Within 3 days of the administration of the irradiated ergosterol these animals developed a considerable diuresis which persisted throughout the subsequent 42 days of the experiment. During this time the extent of the diuresis remained roughly constant and represented an increase of 200 to 300 % on the previous water excretion. The urinary phosphate passed by these animals per rat per day remained almost at its previous level, though still showing considerable daily fluctuations.

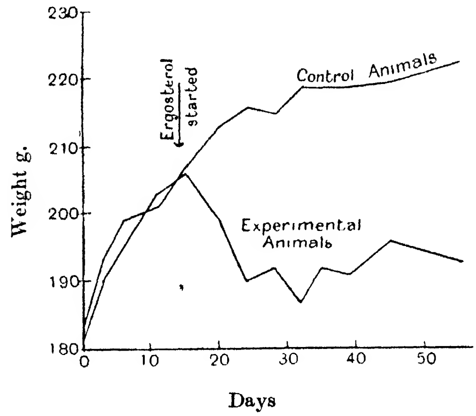

Fig. 1. Showing the average weight curves of the control and experimental animals.

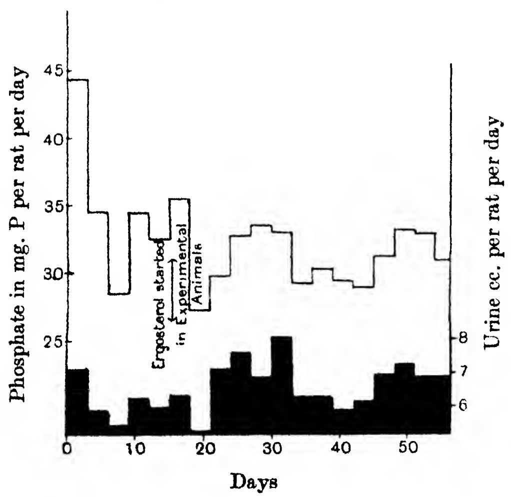

Fig. 2. Showing the urine passed in cc., and the total urinary phosphate in mg. P per rat per day. The two curves are based upon average figures from 12 control animals.

During the last 9 days of the experiment urinary chloride and p_H determinations were made. The figures in Table I show that the total chloride and phosphate passed per rat per day during this period by the control and

experimental groups show no significant differences. The average values of the p_H determinations were also similar.

Table I.

The figures in column 1 are the arithmetic means of the individual readings.
Column 2 is calculated from the formula used by Hill, Long and Lupton [1924].
Column 3 expresses as a percentage the largest observed discrepancy between an individual reading and the mean.
Column 4 expresses as a percentage ratio the difference between the mean values for the control and experimental animals.
Phosphate expressed in mg. P; chlorides expressed in mg. Cl.

		1	2	3	4
		Mean per rat per day	Probable percentage error of mean (calculated)	Maximum percentage error of mean (observed)	Percentage deviation of experimental mean from control mean
Urine	Controls	7·06 cc.	2·8	21	151
	Experimentals	17·8	3·3	26	
Phosphate	Controls	32·5 mg.	2·4	17	17
	Experimentals	38·0	4·8	32	
Chlorides	Controls	44·0 mg.	3·8	23	17·5
	Experimentals	52·8	3·6	26	
p_H	Controls	6·21	3·5	31	4·7
	Experimentals	6·5	3·2	26	

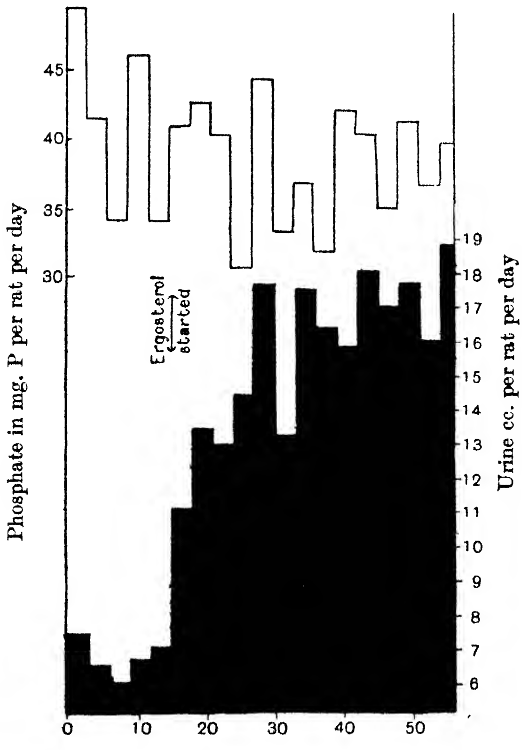

Fig. 3. Showing the urine passed in cc., and the total urinary phosphate in mg. P per rat per day. The two curves are based upon average figures from 12 experimental animals.

Towards the end of the experiment several of both the control and experimental rats were X-rayed; only in one of the experimental animals was a calculus defined with certainty, and in this case a calculus, some 4 mm. in diameter on the plate, was observed in the bladder. At the end of the experiment all the rats were killed and examined *post mortem*. No pathological changes were observed in any of the control animals. In all the experimental rats the kidneys presented a streaky, yellowish white appearance at the cortico-medullary junction, in some cases extending into the papilla, similar to that already reported by Dixon and Hoyle. In nine of these rats calcium phosphate calculi were found in the kidneys, ureters or bladder. They varied in number, size and position, but the frequency of secondary mechanical changes was not so marked as in the former experiments of Dixon and Hoyle. In no case was there evidence of gross infection of the urinary tract. In four animals there was some degree of arteriosclerosis of the thoracic or abdominal aortae. In all these cases the other main arteries showed no lesions.

Discussion.

This experiment affords complete confirmation of the results already published by Dixon and Hoyle. In no case did the irradiated ergosterol administered prove fatal to the experimental animals, and the weight changes and *post mortem* findings confirm their results except in the presence, in four of our experimental animals, of some degree of arteriosclerosis in the aorta. As this was present in a few animals only, and was limited in distribution even in these, and as further the experiment had been continued about four times longer than in the case of those reported by the German workers, it is evident that the arterial changes found in the present case have considerably less significance.

All the experimental animals showed a persistent diuresis without any significant increase in either the total phosphate or chloride output in the urine, so that the concentrations at which these substances were passed varied inversely to the extent of the diuresis. As there was no increase in the total phosphate output this substance was not the cause of the diuresis, since, with a low threshold substance such as this, a diuresis directly due to it causes an increase in total output without any marked fall in the concentration at which it is passed. No further light was thrown on the matter by the p_H determinations. The average urinary p_H of the experimental animals was slightly more alkaline than that of the controls, as would be expected with a diuresis. The figures show that there is no evidence that phosphate is precipitated in the urinary tract as the result of a marked change of p_H towards the alkaline side. Moreover, during the diuresis the total outputs of phosphate and chloride, and the relative concentrations at which they were passed, followed an approximately parallel course. This shows that there is no evidence of any selective effect on the tubular reabsorption of phosphate.

The cause of the diuresis remains at present unexplained. It may well be that a marked increase in the urinary calcium excretion is the determining factor. If this is not so, the occurrence of urinary calculi composed of calcium phosphate in animals that have exhibited a diuresis in which the urinary phosphate concentration is always less than half the normal, without any increase in the total phosphate passed and without any change in the reaction of the urine, must be a very unusual condition.

Conclusions.

1. Confirmation of the effects described by Dixon and Hoyle as occurring in rats given large doses of irradiated ergosterol have been obtained. Emphasis is laid on the non-lethal effects, the absence of persistent or marked loss in weight, and the presence of urinary calculi *post mortem* as the main features of such experiments when ergosterol that has been irradiated in oily solution is used.

2. Such animals show a persistent diuresis without any increase in the total phosphate and chloride passed per day, and without any significant change in the reaction of the urine.

The work in this paper has been done during the tenure by one of us (J. C. H.) of the Ernest Hart Memorial Scholarship of the British Medical Association.

Note (added 11. v. 29, Ed.).

In a paper published since this was sent to press, Harris and Moore [1929] have confirmed their previous findings with ergosterol irradiated in alcohol, and have further found similar effects with ergosterol irradiated in oil. In both cases the *post-mortem* findings were identical with those described by the German workers, with in addition atrophy of the thymus, and in many cases the presence of urinary calculi. Further, they found that ergosterol which had been over-irradiated was completely innocuous, and on the basis of these results they consider that the suggestion made by Dixon and Hoyle, that some additional toxic factor dependent upon the method of irradiation is responsible for poisonous effects, is not valid. If their findings are confirmed this must be so. But in the experiments described in our paper no such results have been found.

Harris and Moore suggest that the reason why Dixon and Hoyle failed to obtain the toxic effects in full measure is because insufficient irradiated ergosterol was given, or that it was not administered for a sufficient length of time. We do not think that this can be the explanation. In the first place, Harris and Moore have obtained the full toxic effects in about 40 days when the equivalent of only 1 to 2 mg. of ergosterol irradiated in alcohol was given daily, as "radiostol," *i.e.* irradiated in oil. Assuming that this ergosterol was of maximal antirachitic activity—that is, about 10,000 units per mg.—the daily dose would be some 20,000 antirachitic units. In Dixon and Hoyle's experiments up to

17 mg. daily were given, and in the experiments in the present paper an average of 24 mg. daily, in both cases for the same period of time as in Harris and Moore's experiments. Although this particular sample was not tested for antirachitic activity, samples irradiated under exactly similar conditions for the same length of time have shown an antirachitic activity of 1600 units per mg. In our experiments, therefore, some 30,000 to 40,000 units at least were given daily. As the samples used by Harris and Moore were irradiated under similar conditions to ours it is improbable that they were of maximal antirachitic activity, and although these writers state that they were assayed they do not give the values found.

The cause of the difference in experimental findings has yet to be determined.

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LXIV. THE EFFECT OF PARTIAL DECAY ON THE ALKALI SOLUBILITY OF WOOD.

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INTRODUCTION.

IT is generally accepted that at least two types of wood decay are caused by fungi, namely the "brown" and "white" rots. In the former type preferential attack is made on the carbohydrate components of wood substance, and lignin is in the main unaffected, the decayed residue being usually brown in colour. In the latter type lignin seems to be the main objective of the fungus, and in the decayed residue there remain patches or "pockets" of a white substance reputed to consist of pure cellulose. The type names are thus derived from the colour of the residues after attack.

In brown rots it has been shown by the work of Bray and Andrews [1924] that the causal fungi are consistent in their preferential attack on the carbohydrate components, but it cannot be said of the white rots that they are consistent in that lignin is always attacked in preference to carbohydrates. Only a few fungi of the white rot type have been studied in any detail, however, but Hawley and Wise [1926, 1] point out that while one of them, *Trametes pini*, may show a preference for lignin, another, *Polyporus hirsutus*, has a serious effect on the cellulose of the wood of white spruce. It is clear, therefore, that the terms "brown" and "white," as applied to different types of decay, can be accepted in an approximate sense only, since inconsistencies are encountered, in the case of the white rots at least, when the chemical effects on the host are taken into consideration.

A classification of decay types based on the chemical effects of the fungus on the host is suggested in the work of Falck and Haag [1927], Wehmer [1927], and Bavendamm [1927]. These authors favour the use of the terms "destruction" and "corrosion" respectively, the former type to include all forms of fungal decay in which carbohydrates are preferentially attacked, and the latter to include the forms in which lignin is preferentially attacked. According to Wehmer [1927], the "destruction" type is brought about by hydrolysis of carbohydrates, and in the "corrosion" type lignin is depleted by a process of oxidation. Hawley and Wise [1926, 2], however, are not disposed to consider decay purely as one chemical process, on the grounds that any decomposition of a nature so profound as the fungal decay of wood could not strictly be compared to a single type of chemical reaction.

In much of the work already done on the chemistry of decay, material has been examined in which decay has been allowed to proceed to a considerable extent, and there is little doubt that in its advanced stages fungal decay is a profound decomposition of a nature difficult of diagnosis by present methods of analysis. At the same time, however, there is evidence that decay takes place in stages [Hawley and Wise, 1926, 3], and the possibility must not be overlooked that, in the very early stages, reactions of a comparatively simple nature may be involved. Also, for all practical purposes the initial stages of decay are the most important, since it is against these that all preservative treatments must be applied. It is obvious, therefore, that a closer understanding of the mechanism of incipient decay should lead to better methods in wood preservation.

The one outstanding feature with regard to fungal decay in wood is that, as a direct result of infection, the residue is rendered more soluble in sodium hydroxide than the original sound wood. In fact Bray [1924] has pointed out that in the case of the brown rots, increase in alkali solubility serves as an index of the extent of cellulose depletion in any given sample of decayed wood. It has also to be noted that acid hydrolysis has the effect of increasing the alkali solubility of wood, and this fact has led Hawley and Campbell [1927] to conclude that there must exist a relationship between acid hydrolysis on the one hand and fungal decay on the other. That fungi are capable of producing acid in various media has been shown by Curtin [1927], and this further strengthens the contention of the above authors, since in the course of its action on wood the fungus may produce acid whereby hydrolysis is brought about. The present investigation has been undertaken in order to obtain a closer comparison between acid hydrolysis and decay than has hitherto been available.

Experimental.

The wood used was Sitka spruce heartwood sawdust of 60–80 mesh. The analytical methods were those recommended by Schorger [1926] and Hawley and Wise [1926, 4].

The general scheme employed was the same as that used by Hawley and Campbell [1927] in their study of acid hydrolysis. Analytical data were obtained for the main constituents of the original wood, and the effect of 1 % sodium hydroxide at 100° was determined on these as before. The results are given in Tables I and II.

Table I. *Analysis of original wood.*

Results on basis of weight of original dry wood.

Cold water-soluble	1·6 %
Hot water-soluble	3·6
1 % alkali-soluble	12·6
Cellulose	62·6
Lignin	26·2
Methoxyl	4·7
Total pentosans	8·4
Pentosans not in cellulose	5·1

Table II. *Analysis of residue after treatment with* 1 % *NaOH for* 1 *hour at* 100°.

Results expressed as percentages by weight of original dry wood.

Loss on alkali treatment	13·8
Cellulose	59·0
Lignin	24·3
Methoxyl	4·0
Total pentosans	6·8
Pentosans not in cellulose	3·9

In studying the effect of decay on alkali solubility, samples of decayed wood were analysed, treated with sodium hydroxide, and the residues analysed. The experiments were carried out in the following manner.

Three separate weighed samples of the 60–80 mesh material were placed in flasks, and moistened with water. The flasks were then plugged with cotton wool and sterilisation was effected by steaming for 30 minutes on each of three consecutive days. After inoculation with *Trametes serialis* Fr. one sample was left to decay for 3 months, and the other two for 4 months at 20°.

After decay each sample was collected on a linen filter and washed with cold water until free from acid. Drying was effected by suction as far as possible, and then, to arrest decay, the samples were further dried and sterilised by heating in weighing bottles at 105° for 16 hours. After cooling in desiccators the samples were weighed, and the loss in weight sustained by each was determined.

Each sample was now divided into two portions, one to be analysed without delay, and the second after treatment with 1 % sodium hydroxide for 1 hour at 100°. The analytical results are given in Tables III and IV.

Table III. *Analysis of residues after decay and washing with cold water.*

Results expressed as percentages by weight of original dry wood.

Duration of decay	Loss due to decay plus cold water washing	Hot water-soluble	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose
3 months	6·98	2·3	55·5	25·8	4·3	7·2	4·8
4 months (1)	8·0	2·9	53·6	25·9	4·2	6·9	4·7
4 months (2)	8·96	3·4	52·7	25·9	4·2	6·8	4·7

Table IV. *Analysis of residues after decay, washing with cold water, and subsequent treatment with* 1 % *NaOH for* 1 *hour at* 100°.

Results expressed as percentages by weight of original dry wood.

	Loss due to decay plus cold water washing	Loss on alkali treatment	Total loss	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose
(1)	6·98	23·1	30·08	44·7	22·6	3·6	4·6	2·6
(2)	8·0	22·4	30·3	42·9	23·0	3·5	4·4	2·4
(3)	8·96	24·9	33·86	40·0	22·5	3·5	4·1	2·4

Discussion.

As previously mentioned a steaming treatment was applied prior to inoculation of the wood with the fungus. That such a treatment has no detrimental effect on spruce wood has been shown by Hawley, Fleck and Richards [1928].

The results in Table III were obtained after the decayed residues had been washed with cold water and heated for 16 hours at 105°. These steps were considered necessary for the following reasons.

In the first place the decayed residues were found to be distinctly acid, hence it was imperative that all traces of acid should be removed prior to analysis. The loss due to decay and the cold water-soluble material was therefore determined as a whole. That some water-soluble material remained in the residues after the preliminary washing was proved by carrying out a hot water-soluble determination on each sample. Therefore, although the exact loss due to decay itself has not been determined, its effect on the wood can be visualised by a comparison of Tables I and III, for, even if all the water-soluble material had been left in the residues, it is certain to have been lost during subsequent analysis, and thus the analytical data would have been the same as those recorded.

As to the heat treatment applied after washing and prior to analysis, this was considered to be the only efficient means of arresting decay at the required time, and of getting the material into a convenient state for analysis. Peterson and Bray [1928] have pointed out that an oven-drying treatment has the effect of lowering cellulose yields, and of rendering the isolation of cellulose more difficult, but in this investigation no difficulties were experienced in isolating cellulose with a normal amount of chlorination after heating the residues for the time stated.

Fungal mycelium, if present in considerable amount in decayed residues, would tend to vitiate analytical results, but attempts to apply corrections for this source of error have proved unsatisfactory. In any case, where decay is only allowed to proceed for a short period, the actual amount of mycelium in the infected material could not be sufficient to detract from the purely comparative value of the analytical data. Care was taken thoroughly to mix the decayed material so that mycelial remains should be distributed as evenly as possible throughout the whole.

Comparison of Tables I and III shows that the fungus has behaved as a typical brown rot in the manner of its attack on wood. Cellulose and pentosans have been depleted, and lignin has been affected to a slight extent, the comparatively small loss in lignin being consistent with a corresponding fall in the methoxyl content. Moreover, it can be seen that the pentosan losses are chiefly due to depletion of the pentosans in the cellulose. That such behaviour is typical of brown rots has been amply demonstrated in previous work, but it was necessary to obtain these results for comparison with Table IV which

shows, firstly, the marked increase in alkali solubility due to decay, and secondly, the detailed effect of alkali treatment on decayed wood.

The increase in alkali solubility is largely accounted for by losses in cellulose and pentosans, the lignin in decayed wood being only slightly more soluble in alkali than the lignin in sound wood.

Whereas in Table I the sum of the four main constituents—namely water-soluble material, cellulose, lignin, and pentosans not in the cellulose—amounts to 99·1 % of the original wood, the sums of the main constituents in the samples of decayed wood, inclusive of the losses due to decay and water-soluble material, amount to 95·58, 95·1, and 95·66 % of the original wood respectively. These facts are in agreement with previous work in that they illustrate that there is in decayed wood a material which is insoluble in water, and is not determined as cellulose, lignin, or pentosans. That such material is probably a carbohydrate degradation product is illustrated by the fact that only the carbohydrates in the wood have been seriously affected by decay. Further, in Table IV, the sums of the main constituents, including the losses due to decay and alkali treatment, amount to 99·98, 98·7, and 98·76 % respectively of the original wood. The fact that these totals are approximately equal to the sum of the main constituents in the original wood indicates that, whatever the material undetermined in the decayed wood may be, it is largely soluble in 1 % sodium hydroxide.

Conclusions.

In comparing the foregoing experimental data with regard to decay and its effect on the alkali solubility of wood with those cited by Hawley and Campbell [1927] in their study of acid hydrolysis, a marked similarity in effect between the two processes is apparent.

The fact that both decayed and hydrolysed wood contain a material insoluble in water, but soluble in 1 % sodium hydroxide, which cannot be determined as cellulose, lignin, or pentosans, tends to strengthen the conviction that, in its early stages at least, fungal decay of the brown rot type is in effect an acid hydrolysis. The only possible objection to this conclusion is to be found in the apparently greater solubility of decayed wood in alkali. Hawley and Campbell [1927] emphasise that the alkali solubility of decayed wood is always greater than that of wood that has been hydrolysed to the same extent (as shown by equal loss in weight) but, all things considered, this is only to be expected. Whereas in the hydrolysis of wood with dilute acid all the water-soluble material is removed, wood that has been decayed still contains water-soluble material. Where the losses in weight are the same the alkali solubility of decayed wood will always be greater than that of hydrolysed wood in proportion to the amount of water-soluble material present in the former. If, in Table III, the percentage of hot water-soluble material be added to the loss sustained by decay plus cold water washing, and in Table IV deducted from the loss on alkali treatment, decay can be

strictly compared with hydrolysis. This can be illustrated by the following example.

In Table III the loss in weight sustained by one of the samples after 4 months' decay and cold water washing was 8·0 %. If the figure for the remaining water-soluble material be added to this a total of 10·9 % is obtained. This total is approximately equal to the loss obtained by Hawley and Campbell [1927] by hydrolysing Sitka spruce with 0·25 % hydrochloric acid. Furthermore, the losses due to decay, as expressed by a comparison of Tables I and III, agree very favourably with the losses involved in a hydrolysis with acid of the above concentration. Again, if in Table IV the amount of hot water-soluble material be subtracted from the loss due to alkali extraction, this latter is reduced from 22·4 to 19·5 % of the weight of the original wood. Hawley and Campbell [1927] have shown that Sitka spruce wood which has been hydrolysed with 0·25 % hydrochloric acid is soluble in 1 % sodium hydroxide to the extent of 18·2 %. Thus it can be seen that the agreement between the degree of alkali solubility of partially hydrolysed wood and that of partially decayed wood is in reality quite close, when the necessary allowance is made for the amount of water-soluble material in the latter.

With the advanced stages of decay this investigation is not strictly concerned, but it seems probable that the high degree of alkali solubility of the lignin in badly decayed wood is due, not so much to any direct effect of decay on the lignin complex, as to the large increase of surface area of the lignin caused by marked depletion of the carbohydrate components of the wood.

From the foregoing considerations the similarity of the effect on wood between acid hydrolysis on the one hand, and fungal decay of the brown rot type on the other, is so close as to warrant the conclusion that decay of the brown rot type should, in effect, be regarded as an acid hydrolysis.

Summary.

1. The effect of partial decay on the wood of Sitka spruce caused by *Trametes serialis* Fr. has been examined. This effect is typical of that produced on wood by fungi of the brown rot type.

2. The effect of partial decay on the alkali solubility of the wood of Sitka spruce has been examined, and reasons are given for concluding that, since this effect is of the same order as that produced in the same species of wood by acid hydrolysis, decay of the brown rot type should, in effect, be regarded as an acid hydrolysis.

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LXV. THE NATURE OF THE SUGAR RESIDUE IN THE HEXOSEMONOPHOSPHORIC ACID OF MUSCLE.

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Embden and Zimmermann [1924] have described the isolation from press-juice of rabbit muscle of a hexosediphosphoric acid which was obtained in the form of its neutral brucine salt. They experienced some difficulty in attempting its isolation owing to the presence of free phosphoric acid in their press-juice. Ultimately they eliminated the free phosphoric acid by employing a biological synthetic process involving the use of the fluoride ion which is known to further the synthesis of hexosephosphoric acid from carbohydrate and phosphates. Later Embden and Zimmermann [1927, 1], employing a somewhat different procedure and omitting the fermentative resynthesis, obtained from rabbit muscle pulp a hexosemonophosphoric acid. They were unable to detect the presence of a hexosediphosphoric acid.

In the present investigation it was desired to obtain a supply of the muscle hexosephosphoric acid (frequently referred to as "lactacidogen") in order to study the nature of the sugar residue present in this interesting intermediate substance. The two methods of Embden and Zimmermann were therefore both investigated as a means of obtaining one or other of the two hexosephosphoric acids, our immediate purpose being to obtain that which could be demonstrated to be a normal constituent of the muscle.

The earlier work of Embden and Zimmermann has been fully confirmed and we have obtained from rabbit muscle press-juice the neutral brucine salt of hexosediphosphoric acid identical in all respects with that obtainable from yeast fermentation and now shown by Robison and Morgan [1928] and by Morgan [1929] to be γ-fructose-1 : 6-diphosphoric acid. Similarly, employing the later methods of Embden and Zimmermann, we have likewise obtained only a hexosemonophosphoric acid and have been unable to detect the presence of any of the di-acid ester. It seemed possible that the different results obtained in the two processes of extraction might not be wholly ascribable to the omission of the fermentative resynthesis in the later method, but might in part be due to the other modifications introduced at the same time. It was therefore decided to check this point by employing the first method of extraction, omitting only the addition of sodium fluoride, glycogen and sodium bicarbonate, the reagents used in the fermentative resynthesis. There was

obtained only a hexosemonophosphate identical with the product obtained by the second method of extraction. No trace of diphosphoric acid was detected. The authors are therefore of opinion that the carbohydrate-phosphoric acid ester normally obtainable from resting muscle and regarded as "lactacidogen" consists of hexosemonophosphoric acid. The material used in the present investigation was therefore prepared from the mono-acid ester obtained by Embden and Zimmermann's second method of extraction.

Hexosemonophosphoric acid has been isolated not only from the muscles of rabbits but also from those of the goat and donkey. The yields obtained from the latter sources were lower than those obtained from the rabbit, details being given in the experimental section. This may in part be due to the enzymic breakdown of the compound owing to the necessarily longer time required for the preliminary extraction, but it is our opinion that the poorer yields cannot be wholly ascribed to this cause. The extraction was carried out with all possible speed and the cooling arrangements were as efficient as in the rabbit muscle extractions. It appears possible that the hexosemonophosphoric acid content of the muscles of the larger and more slowly moving animals may normally be less than that of the rapidly contracting muscles of the rabbit. During the course of the rabbit muscle extractions adenylic acid was obtained as has been recorded by Embden and Zimmermann [1927, 2], but this compound was obtained only in very small yield from the muscles of the larger animals. On the other hand, considerable quantities of inositol were obtained from the muscles of the goat and donkey, whilst it was not isolated from any of the rabbit muscle extracts. The best yield of hexosemonophosphoric acid obtained in the present work was 9 g. (37 g. of the neutral brucine salt) from 7 kg. of rabbit muscle, corresponding to 0·13 % of the weight of fresh muscle.

In the experimental section there is described the preparation of free hexosemonophosphoric acid *via* the barium salt, from the recrystallised brucine salt in which form it was first isolated. The acid showed $[\alpha]_{5461} + 33{\cdot}6°$, which is in good agreement with that quoted by Embden and Zimmermann for their preparation, namely $[\alpha]_D + 29{\cdot}5°$. Employing the Willstätter-Schudel hypoiodite method of oxidation these workers found that the sugar constituent of their acid consisted of 91 % of aldose in one preparation and 93 % of aldose in another. Our preparations showed 90 % of aldose sugar. From the free acid a crystalline osazone was obtained without loss of the phosphoric acid grouping. It melted in the vicinity of 145–147° with decomposition.

After a series of preliminary investigations which need not be detailed here it was decided that the nature of the sugar residue present in muscle hexosemonophosphoric acid could best be decided by oxidation to the corresponding hexonic acid and subsequent removal of the phosphoric acid group. The first step was effected with the use of bromine and the second with 10 % sulphuric acid in a sealed tube at 100°. There was finally obtained the calcium salt of a hexonic acid which was free from phosphorus and from reducing material. The free hexonic acid prepared from the calcium salt had $[\alpha]_{5461} + 10{\cdot}9°$, and

after heating for 1 hour at 65°–70° it showed $[\alpha]_{5461} + 24{\cdot}0°$. This change is characteristic of a hexonic acid undergoing lactonisation and these figures are in good agreement with those obtained for pure preparations of gluconic acid subjected to the same treatment. We are therefore of the opinion that the aldose of hexosemonophosphoric acid is *d*-glucose.

Experimental.

Isolation of hexosediphosphoric acid from rabbit muscle press-juice.

The first pair of rabbits used were not specially fed, but in the later experiments the rabbits were fed with generous supplies of oats for at least 3 days immediately before they were killed. Some were killed by a sharp blow on the back of the neck followed by immediate decapitation. A disadvantage of this method of killing is the resulting violent twitching of the muscles, and in the later experiments the animals were asphyxiated with coal gas until the corneal reflex failed and then decapitated. The pelt was removed as quickly as possible and the musculature was excised and minced in an ice-cold machine. The subsequent steps in the extraction were essentially those of Embden and Zimmermann [1924]. A Buchner oil press was used, and the juice was collected under pressures up to 150 kg./cm.^{2} The cloth used was made of camel hair and was supplied by Messrs Premier Filterpress Co., Ltd., London. The yields obtained are indicated by the following typical results.

I. 2 rabbits, rather small and not specially fed. Juice obtained, 415 cc.; recrystallised brucine salt, 1·5 g.

II. 2 rabbits, fed on previous day with oats. Juice obtained, 500 cc.; recrystallised brucine salt, 2·5 g.

III. 2 rabbits, fed for 3 days with oats. Juice obtained, 540 cc.; recrystallised brucine salt, 3·5 g.

The brucine salt was twice recrystallised from aqueous methyl alcohol and dried *in vacuo* over sulphuric acid and finally at 56° over phosphorus pentoxide in a high vacuum. It gave satisfactory analyses and showed $[\alpha]^{17°}_{5461} - 30{\cdot}7°$ ($c = 1{\cdot}01$ %). For comparison purposes Mr W. J. T. Morgan, of the Lister Institute, London, kindly prepared a specimen of brucine hexosediphosphate from yeast fermentation and this showed in methyl alcohol $[\alpha]^{18°}_{5461} - 30{\cdot}7°$, thus confirming the identity of the two products. When heated rapidly our twice recrystallised preparation melted with considerable charring between 170° and 180°.

Isolation of hexosemonophosphoric acid from the press-juice of rabbit muscle.

For reasons already given in the introduction it was decided to isolate from rabbit muscle and identify the hexosephosphoric acid or acids, using the same method as that used for the isolation of hexosediphosphoric acid, omitting only

the fermentative resynthesis involving the addition of glycogen and sodium fluoride in the presence of sodium bicarbonate.

The muscle juice from six normal rabbits was collected in as short a time as possible and in efficiently cooled vessels. With one exception, when the rabbit was killed by a sharp blow, all the animals were asphyxiated with coal gas. They had all been previously fed on oats for at least 3 days. The total volume of juice obtained was 1350 cc. At the stage when deposition of brucine hexosediphosphoric acid should have occurred no deposition of crystals was noted even after prolonged scratching and on allowing the solution to stand in the ice-chest overnight. The following day slow crystallisation began and after 2 days some clusters of small crystals separated from the solution. These were obtained in very small amount and were found to contain a pentose. They were subsequently proved to consist of the brucine salt of adenylic acid, to which reference will be made in a later communication. The solution was treated with several volumes of acetone in order to complete the deposition of the brucine salt of adenylic acid. The solid obtained was extracted with a small volume of anhydrous methyl alcohol and filtered. The filtrate was concentrated to dryness and again extracted with methyl alcohol and filtered. The solution was again taken to dryness and the crystalline residue was dried to constant weight. It had $P = 2{\cdot}83\ \%$ (calc. for brucine hexosemonophosphate $2{\cdot}96\ \%$ and for the hexosediphosphate $3{\cdot}21\ \%$), and showed $[\alpha]_{5461} - 19{\cdot}4^\circ$ in methyl alcohol. Our later preparations of brucine hexosemonophosphate showed in the same solvent $[\alpha]_{5461} - 20{\cdot}3^\circ$. In this experiment therefore there was obtained no evidence of the presence of a hexosediphosphoric acid and it is concluded that the latter is either entirely absent or present only in very small amounts in normal muscle press-juice.

Isolation of hexosemonophosphoric acid from rabbit, goat and donkey muscle.

The method of extraction was that already referred to as Embden and Zimmermann's second method, with minor modifications which need not be detailed.

Embden and Zimmermann [1927, 1] report an average yield of 11 g. of unrecrystallised brucine hexosemonophosphate from twelve rabbits yielding 6 kg. of muscle. The best yield obtained in the present work was 23 g. of brucine hexosemonophosphate from twelve rabbits yielding 7 kg. of muscle, whilst in this case the basic lead acetate fraction gave a further yield of 14 g. of the brucine salt. That our yields are materially higher than those recorded by Embden and Zimmermann is doubtless in part due to the fact that we subjected all protein residues to high pressure extraction, using the Buchner press.

After one recrystallisation brucine hexosemonophosphate gave in water $[\alpha]_{5461} - 29{\cdot}9^\circ$ and in anhydrous methyl alcohol $- 20{\cdot}3^\circ$. The recrystallised salt after drying *in vacuo* over phosphorus pentoxide showed signs of softening at 145°, melted at 155° and decomposed at 158–160°.

In order to obtain larger supplies of hexosemonophosphoric acid extracts were made from the minced muscles of larger animals, namely two goats and one donkey. The yields were disappointingly small considering the size of the animals. The animals were fed for some days on oats and hay and were killed with a "humane killer" and in no case was any muscle twitching observed after the death of the animal. The extractions were carried out in a manner identical with that used in the case of the rabbits and the operations were expeditiously carried through and the bulky solutions were efficiently cooled with ice. The protein precipitates were filtered on large stoneware filters, using muslin to give added support to the filter-papers. The work was continued without a break until the deprotemised filtrates had been freed from mercury and excess hydrogen sulphide and then almost neutralised with 33 % sodium hydroxide. The results may be summarised as follows.

Goat I ♀. All muscles minced and in acid bath in 25 minutes. Temp. rose for few minutes to 10°, but was quickly reduced to 2°. Weight of muscle, 6 kg.

Via normal lead acetate precipitate, 3·3 g. of brucine salt and 1·3 g. inositol.

Via basic lead acetate precipitate, 8·8 g. brucine salt and 1·8 g. inositol.

Goat II ♀. All muscles minced and in bath in 20 minutes. Temp. rose for few minutes to 10°, but solution was quickly cooled to 2°.

Weight of muscle, 10·5 kg.

Via normal lead acetate precipitate, 1·4 g. brucine salt and 0·7 g. inositol.

Via basic lead acetate precipitate, 3·0 g. brucine salt and 1·3 g. inositol.

Donkey ♂. All muscles minced and in acid bath in 50 minutes. Cooling arrangements efficient. Temp. quickly reduced to about 3° or 4°. Weight of muscle, 30·2 kg.

Via normal lead acetate precipitate, 3·4 g. brucine salt and 0·2 g. inositol.

Via basic lead acetate precipitate, 2·1 g. brucine salt and 4·5 g. inositol.

The brucine salts after recrystallisation gave the same optical rotation and melting point as those obtained from rabbit's muscle. Further, the free hexosemonophosphoric acid prepared *via* the barium salt from the mixed brucine salts obtained from rabbit, goat and donkey gave the same optical rotation.

A point which has been referred to in the introduction is the absence of adenylic acid and the presence of inositol in these later extractions. Using Bial's reagent it was established that the pentose-containing constituent was present only in very small amounts and it was found impossible to isolate any adenylic acid either from the goats or from the donkey. On the other hand, there was obtained, from the aqueous-acetone solutions from which adenylic acid was deposited in the case of the rabbit muscle extractions, a white crystalline compound which was recrystallised from hot water, in which it was readily soluble. It gave a negative result on testing with Molisch's reagent, did not reduce Fehling's solution, contained no nitrogen or pentose constituent and possessed a definitely sweet taste. It was found to be optically inactive, which indicated with the previous tests that this crystalline compound was *i*-inositol. This was confirmed by a melting point, the dried substance melting at 224–225°

with preliminary softening, and also by a positive Sherer's test. After two recrystallisations from water the substance still retained a slight trace of organically combined phosphorus (indicated with Bell-Doisy reagents).

Preparation of barium hexosemonophosphate.

Barium hexosemonophosphate was prepared from the recrystallised brucine salt by the method adopted by Embden and Zimmermann. The barium salt was precipitated as an amorphous solid by adding a saturated solution of barium acetate in 80 % methyl alcohol to a 10 % aqueous solution of the brucine salt. After standing for a few hours in the cold the salt was collected on a small Büchner funnel, washed with 80 % methyl alcohol, and finally with pure methyl alcohol. The product was then thoroughly dried over phosphorus pentoxide in a vacuum. It was ground in a mortar with a little water when most of the barium salt dissolved, leaving a less soluble yellowish residue. The barium salt was then reprecipitated with methyl alcohol and treated as before. This treatment was repeated at least once, sometimes twice, with all samples of the barium salt prepared. In this way the final product was freed from brucine and also from the more insoluble constituent with which it is initially associated. In one preparation it was found advantageous to warm for some time with a little norite. Again, in another preparation a considerable quantity of the less soluble material was separated from the barium salt by extracting with warm water. But it is essential that the precipitated product be thoroughly dried before a further aqueous extract is made, otherwise it is impossible to obtain the barium hexosemonophosphate in a state of purity.

Embden and Zimmermann record having obtained evidence of iron and phosphorus in one of the less soluble yellowish residues already referred to. The authors obtained negative results in testing for iron, except in one sample when a very faint blue colour was developed with potassium ferrocyanide, after thoroughly digesting the material with hot concentrated sulphuric acid and 30 % hydrogen peroxide. The brucine used in this instance, however, was itself found to contain traces of iron and it is therefore not thought that the iron found in the residue from the barium hexosemonophosphate has necessarily any biological significance. Repeated extraction of the less soluble material with water left the residues progressively poorer in phosphorus. Further, barium salts (especially those prepared from brucine salts obtained *via* the basic lead acetate precipitates) contaminated with this extraneous material gave low analytical figures and the free hexosemonophosphoric acid subsequently obtained showed a lower specific rotation and gave lower reduction values. These syrupy residues, which on drying form a glass, are therefore not of the nature of sugar phosphates and were not further investigated.

Hexosemonophosphoric acid.

The optical rotation of the free hexosemonophosphoric acid was determined in the following manner. A weighed amount of the dried barium salt was dissolved in water and the barium precipitated by the addition of a slight excess of sulphuric acid. The barium sulphate was centrifuged off, and the clear supernatant solution decanted into a suitable measuring flask. The barium sulphate precipitate was then washed twice with small quantities of water and after centrifuging these were added to the solution, which was finally made up to a definite volume. The free acid, liberated in the above manner from carefully dried barium hexosemonophosphate (Ba = 34·5 %, P = 7·8 %, calc. for $C_6H_{11}O_5(PO_4Ba)$, Ba = 34·7 %, P = 7·85 %), gave $[\alpha]_{5461}^{17°}$ + 33·0° in water (c = 0·770).

The hexosemonophosphoric acid obtained in the same manner from the mixed extracts of rabbit, goat, and donkey muscle, gave $[\alpha]_{5461}$ + 33·6° in water (c = 2·93). As indicated above, specimens of barium hexosemonophosphate which gave low analytical figures, gave rise to a free acid with lower specific rotation and, further, correspondingly low copper reduction values. Thus one preparation of barium hexosemonophosphate gave a specific rotation for the free acid of $[\alpha]_{5461}$ + 23·1 (calc. from the weight of barium salt originally taken). On regrinding some of the barium salt with water a small amount of insoluble material remained. After reprecipitation with methyl alcohol the salt was carefully dried as before. The barium salt obtained still had a low phosphorus content (P = 7·25 % calc. 7·85), but the rotation was higher than the previous value, being $[\alpha]_{5461}$ + 26·5°.

The copper-reduction value of hexosemonophosphoric acid was then determined, employing the method described by Bertrand. The values obtained were compared with those of glucose.

$$\text{Ratio } \frac{\text{Hexosemonophosphoric acid (calc. as hexose)}}{\text{Glucose}} = 0{\cdot}74.$$

Formation of barium methylhexosidemonophosphate.

About 2·1 g. of barium hexosemonophosphate was dissolved as quickly as possible in 55 cc. of anhydrous methyl alcohol containing 1 % hydrochloric acid. Polarimetric observations were made on the solution and the observed rotations in a 1 dm. tube are recorded below. After the first 8 hours the solution was maintained at 25° in a well-stoppered flask. A short time after the first observation was made barium chloride began to separate out and it was then necessary to filter the solution immediately before observing the optical rotation. The solution still reduced Fehling's solution 74 hours after it was prepared. Tested after 97 hours the solution showed no reduction with Fehling's solution. Observed rotations $[\alpha]_{5461}$: + 0·41° (8 hrs.), 0·29° (12 hrs.), 0·38° (32½ hrs.), 0·51° (73½ hrs.), 0·57° (98½ hrs.), 0·59° (121½ hrs.), 0·61° (146 hrs.), 0·64° (168½ hrs.). It will be noticed that initially there is a fall in

the observed rotation followed by an increase, which gradually approaches a maximum. These changes are evidently due to the different rates of formation of the α- and β-isomers of the methylhexosidemonophosphate.

An attempt was made to isolate the barium methylhexosidemonophosphate and then to dephosphate this compound with a preparation of bone enzyme, using the method described by Morgan [1927] and applied by him to the investigation of hexosediphosphoric acid from yeast. But the different solubilities of the corresponding salts of the hexosemonophosphoric acid make this method unsuitable, especially with small amounts of material.

An osazone was prepared from the hexosemonophosphoric acid without liberation of free phosphoric acid. 0·3 g. of the barium salt was treated with a slight excess of sulphuric acid and after removing the precipitated barium sulphate the solution was heated for half an hour in a boiling water-bath with 0·5 g. of phenylhydrazine hydrochloride and 1 g. of sodium acetate. Crystallisation commenced immediately the solution was cooled. Viewed under the microscope the crystals presented the appearance of clusters of flat needles. They began to soften at 140° and melted at 145°–147° with decomposition.

Willstätter-Schudel oxidation.

Employing the technique described by Goebel [1927] for the Willstätter-Schudel oxidation with hypoiodite, hexosemonophosphoric acid gave values indicating that 90 % of the hexose in this acid is of an aldose nature.

After oxidation by this method, the solution still reduced Fehling's solution. This is interpreted as indicating the presence of some 10 % of a ketosemonophosphate admixed with 90 % of aldosemonophosphate.

Preparation of hexonic acid from hexosemonophosphoric acid.

About 0·8 g. of hexosemonophosphoric acid dissolved in 23 cc. of water was treated with 0·75 g. of bromine. After standing for 16 hours at air temperature a portion of the solution, after aerating to free from bromine, still reduced Fehling's solution. The solution was still slightly reducing to Fehling's solution after 9 hours in a water-bath kept at 30°. A micro-determination of sugar was then made employing the method of Shaffer and Hartman. Later determinations, after the solution had been maintained at 30° for a further period of 11 hours, gave a reduction value only slightly less than that obtained previously. From these determinations about 0·04 g. (calc. as glucose) remained unoxidised. It may be noted here that only slight traces of free phosphoric acid could be detected in the solution after the above oxidation. The solution was aerated to free from bromine and then sufficient hydrobromic acid added to give a 14 % solution of the acid. This solution was heated in a sealed tube at 85° for 20 hours. On opening the tube, free phosphoric acid was found to be present in small amount only. Sulphuric acid was then added sufficient to give a 10 % solution of this acid, and then after resealing the solution was

heated for 7 hours at 100°. The resulting perfectly clear solution contained considerable amounts of free phosphoric acid. It was neutralised with silver oxide, and the filtrate subsequently obtained was freed from silver with hydrogen sulphide. After aeration the solution was found to be free from phosphorus, non-reducing, and optically active in the dextro-sense. The solution was heated to 75°, calcium carbonate added, and the solution well stirred for several minutes. After cooling, the solution was filtered and concentrated to dryness at 45°. The solid residue was then extracted with a small volume of hot water and filtered. The filtrate still contained calcium sulphate which was precipitated by the addition of an equal volume of absolute alcohol. After standing for some days the solution was filtered and concentrated under reduced pressure. Calcium sulphate was still present in minute quantities, and the solution was again precipitated by adding several volumes of alcohol. The precipitate was collected and extracted with about 10 cc. of water. The small amount of insoluble material was centrifuged off and the clear supernatant solution again precipitated with several volumes of alcohol. The perfectly white product was collected and dried overnight in an evacuated desiccator containing phosphorus pentoxide. The dried material gave a calcium content of 9·2 % (micro-determination) (calc. for $(C_6H_{11}O_7)_2Ca$, 9·3 %).

0·118 g. of the calcium salt was dissolved in water and one drop of concentrated hydrochloric acid added to liberate the free hexonic acid. The volume was made up to 10 cc. thus giving a 1·06 % solution of the free hexonic acid.

This solution gave $[\alpha]^{18°}_{5461}$ + 10·9°.

After the solution had been maintained at 50° for 1½ hours the rotation increased, and on cooling gave $[\alpha]^{21°}_{5461}$ + 14·6°.

On the addition of a small drop of concentrated hydrochloric acid the rotation dropped immediately to its previous value. The solution was reheated for an hour at 65°–70° and then gave $[\alpha]^{68°}_{5461}$ + 26·8°.

The temperature of the jacketed tube was then speedily reduced to 24°. The solution then showed $[\alpha]^{24°}_{5461}$ + 24·0°.

A reading taken 15 minutes later showed identically the same value. These results are in good agreement with those recorded by Tollens [1914] for gluconic acid. The calcium salt has $[\alpha]_D$ + 10° and prepared directly from the calcium salt the equilibrium mixture of free acid and lactone has $[\alpha]_D$ + 23·4° after the solution has been heated for some time.

Summary.

(1) The hexosephosphoric acid of normal muscle press-juice from rabbit, goat and donkey is a monophosphoric acid. The diphosphoric acid, identical with that of yeast fermentation, is only obtained when the fermentative resynthesis using sodium fluoride is employed.

(2) The carbohydrate residue of muscle hexosemonophosphoric acid consists of 90 % of aldose, identified as *d*-glucose, and 10 % of ketose.

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LXVI. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

IV. A COMPARISON BETWEEN GLUCOSE AND GLYCOGEN IN RESPECT OF LACTIC ACID FORMATION AND PHOSPHORIC ESTER ACCUMULATION.

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IN this paper are described eight experiments with individual extracts prepared in the same manner from eight healthy cats. In each case the reaction at the start of the incubation was between p_H 6·9 and 7·1; glucose and glycogen were used respectively in a concentration of 0·5 %. These eight experiments thus constitute a good series for the purpose of detecting any difference between glucose and glycogen with regard to the apparently related reactions of lactic acid formation and phosphoric ester accumulation.

EXPERIMENTAL.

The extracts were prepared from perfused muscle by the method described in a previous paper [Stiven, 1928]. The incubation was conducted in an atmosphere of nitrogen as described therein. The purity of the glycogen was estimated by hydrolysis to glucose and in weighing out the glycogen for making up the stock 10 % solution to be added to the extract, a correction was made for the purity as indicated by the yield of glucose. The lactic acid was estimated by the aeration method of Friedemann, Cotonio and Shaffer [1927]; the "inorganic" phosphorus by the colorimetric method of Fiske and Subbarow [1925]. Both lactic acid and "inorganic" phosphorus were estimated at intervals of 10 minutes up to the end of the first hour and thereafter at 90 minutes and 120 minutes from the start of the incubation. In this way the course of the reactions could be followed at short intervals and any resemblance or difference between the behaviour of these two carbohydrates could be detected.

The term "inorganic" phosphorus means in this paper the sum of the phosphagen phosphorus and the true inorganic phosphorus. In every experiment the lactic acid and the "inorganic" phosphorus changes were investigated in an aliquot sample of the extract to which no carbohydrate had been

added. In this "plain" extract the "inorganic" phosphorus increases rapidly during the first 40 minutes of the incubation but thereafter very slowly. In the case of the extracts to which carbohydrate was added this increase of "inorganic" phosphorus does not take place until the incubation has proceeded for 40 to 60 minutes if lactic acid is being formed from the added carbohydrate. In the early period of the incubation a decrease of the "inorganic" phosphorus may in some cases be observed. This decrease of "inorganic" phosphorus indicates that the formation of phosphoric ester is proceeding at a greater rate than the breakdown of the ester. The amount of this decrease between any two intervals is thus a measure of the excess of ester formed over that broken down in that interval and is called here ester accumulation.

Table I. *Lactic acid formation and phosphoric ester accumulation from glycogen and glucose both present to* 0·5 %.

Experiment		Lactic acid formed (mg. per 100 cc. extract) in 20 mins.	40 mins.	60 mins.	120 mins.	Maximum decrease of "inorganic" phosphorus (mg. per 100 cc. extract)	Time of maximum ester accumulation (mins.)
252	Glycogen	106	170	227	264	21	10
	Glucose	118	213	300	427	3	10
254	Glycogen	72	98	114	130	25	20
	Glucose	78	137	162	186	2	20
255	Glycogen	80	143	171	207	35	40
	Glucose	84	162	224	295	0	—
256	Glycogen	107	157	195	284	29	10
	Glucose	70	141	202	321	2	60
257	Glycogen	102	154	191	233	13	10
	Glucose	64	104	113	119	0	—
260	Glycogen	127	183	229	260	38	20
	Glucose	145	229	247	250	1·5	10
261	Glycogen	101	154	192	301	46	40
	Glucose	79	125	192	332	1·5	50
262	Glycogen	90	167	216	252	6	10
	Glucose	69	150	222	350	0	—

From the results in Table I it will be noticed that decidedly greater ester accumulation took place in every case with glycogen. With glucose it was very feeble and in some cases never took place at all. The striking difference between glucose and glycogen in this respect is not due to a less extensive formation of lactic acid from the hexose nor to a lower rate of formation of the acid. These results seem to indicate that at the start of the incubation, when the concentration of the glycogen is high, the formation of phosphoric ester generally proceeds at a greater rate than the subsequent removal of the phosphoric acid groups. With glucose, on the other hand, it would appear that these two opposing and concomitant reactions almost balance, and from an examination of a great many extracts it has been noticed that the greater the rate of glycolysis the more does the rate of ester formation tend to exceed that of the breakdown. If lactic acid originates only from carbohydrate that

has been through the intermediate stage of phosphoric ester, then it is difficult to understand why in the case of glycogen the ester should sometimes accumulate to the extent it does, particularly in those cases where the rate of lactic acid formation is greater than with glucose, indicating to all appearances a more rapid hydrolysis of the ester. It is conceivable, however, that the ester which accumulates in the glycogen samples does not have the same structure and hence does not yield the same end product as the intermediate ester formed from glucose. This point is under investigation and will be discussed in subsequent papers.

Another point deserves consideration. It will be noticed that the lactic acid formation from glucose varies greatly from one experiment to another. In some cases it is greater with glucose than with glycogen and in other cases it is not. It might be argued that in a series of experiments like the above where the conditions were kept as uniform as possible, this variation might be due rather to faulty technique than to a real difference produced by some unknown factor. The following experiment shown in Table II tests this point. 2 kg. of cat muscle were ground up and extracted in the usual manner, and the final volume of extract, 900 cc., was divided up into three portions of 180 cc. To each of these were added 9·5 cc. of 10 % glucose solution. The "inorganic" phosphorus and the lactic acid were estimated at the intervals indicated.

Table II, Exp. No. 265. *"Inorganic" phosphorus concentrations and lactic acid increases in three aliquot portions of the same extract, all containing* 0·5 % *glucose.*

Time in mins.	"Inorganic" phosphorus concentration (mg. per 100 cc.)			Increase in lactic acid (mg. per 100 cc.)		
	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C
Start	71·3	71·0	71·0	—	—	—
10	70·5	70·5	70·5	36	36	38
20	71·0	71·0	71·0	72	75	72
40	72·3	72·3	72·1	166	167	166
60	75·7	76·0	76·0	219	222	228
90	82·3	82·9	82·9	291	308	311
120	89·5	89·5	89·5	313	325	327

The above results indicate the extent of the variation that is to be expected. The lactic acid values show greater fluctuations than the phosphorus values but these are not of sufficient magnitude to account for the erratic nature of the results in Table I. These must be due to some unknown factor connected with the preparation of the extract or even with the animals. In this connection it has been found that tame rabbits, as compared with wild rabbits or cats, give invariably most disappointing results as regards lactic acid formation from glucose.

Summary.

Extracts from the muscles of eight cats have been examined for lactic acid formation and phosphoric ester accumulation from both glucose and glycogen. In the case of glucose the extent of the ester accumulation is very small compared with that from glycogen even when the rate of lactic acid formation from glucose is greater than from glycogen. Both the rate and extent of the lactic acid formation from glucose are, generally speaking, greater than from glycogen, but in this respect there is a great variation from one extract to another. It is shown that this variation is not due to faulty technique in the manner of incubation or in the analytical procedure, but is due to some factor as yet uncontrolled.

In concluding, the author wishes to express his thanks to Prof. E. Waymouth Reid, F.R.S., for his assistance in the perfusing of the cats.

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LXVII. THE DETERMINATION OF THE TERTIARY DISSOCIATION CONSTANT OF PHOSPHORIC ACID.

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QUANTITATIVE studies of the mechanism of calcification require a knowledge of the physico-chemical constants of the blood equilibria involved in the process [Kugelmass, 1924; Kugelmass and Shohl, 1924].

The values for the tertiary dissociation constant of phosphoric acid available at present are at variance. Abbott and Bray [1909] found K_3 to be $3{\cdot}6 \times 10^{-13}$ at 18° for a total concentration of 0·026 *M* by means of conductivity and distribution ratios of ammonia between chloroform and solutions of sodium ammonium phosphate. This value seems incompatible with the apparent dissociation constant p_{K_3}, from the dissociation curve for the phosphates, which is of a higher order of magnitude.

The tertiary dissociation constant of phosphoric acid was determined from a study of the aqueous systems of the tertiary phosphates at equilibrium from the standpoint of hydrolysis.

The neutralisation of orthophosphoric acid by strong alkali has been studied by means of thermochemical, electrometrical, and conductance measurements. Thomsen has shown that the small heat effects that attend the addition of the third equivalent of base indicate that the third equivalent of hydrogen of the acid is far from completely replaced when the proportions of acid and base are those corresponding to the salt Na_3PO_4. Berthelot found that the decrease of conductance during neutralisation may be represented by a straight line up to the point at which the first hydrogen of the acid is replaced and by another straight line different in direction from the first up to the point corresponding to the replacement of the second hydrogen. Beyond this point further addition of alkali causes a change in the direction of the curve, which can no longer be represented by a straight line. Hence, the first and second equivalents of hydrogen in orthophosphoric acid are almost quantitatively replaced but the third equivalent is only gradually neutralised as the proportion of base increases. These results clearly indicate that the tertiary phosphate is hydrolysed to a considerable extent.

THEORETICAL.

Derivation of equations. The tertiary dissociation constant may be evaluated from the degrees of hydrolysis of the tertiary phosphates. The hydrolysis occurs progressively in several stages, to each of which correspond definite equilibria represented by the following stoichiometric equations:

$$\begin{aligned} Na_3PO_4 + H_2O &\rightleftharpoons Na_2HPO_4 + NaOH \quad \ldots\ldots(1). \\ Na_2HPO_4 + H_2O &\rightleftharpoons NaH_2PO_4 + NaOH \\ NaH_2PO_4 + H_2O &\rightleftharpoons H_3PO_4 + NaOH \end{aligned}$$

The tertiary phosphate hydrolyses to a great extent, the secondary and primary salts inappreciably, as will be evident from the data below. Such an aqueous system probably also contains the intermediate ions, $Na_2PO_4^-$ and $NaPO_4^=$, but we know very little about their concentrations or activities. Hence, the first stage hydrolysis (equation (1)) may be considered as the essential equilibrium.

The mechanism of equation (1) may be expressed by the ionic equilibrium:

$$PO_4^{\equiv} + H_2O \rightleftharpoons HPO_4^= + OH^-.$$

Applying the law of mass action,

$$\frac{[HPO_4^=][OH^-]}{[PO_4^{\equiv}]} = K_h \quad \ldots\ldots(2),$$

where the brackets include the ion activities; K_h is the "activity" hydrolysis constant.

The degree of hydrolysis, h, is by definition,

$$h = \frac{[HPO_4^=]}{\gamma_S C} = \frac{[OH^-]}{\gamma_B C} \quad \ldots\ldots(3)$$

and

$$1 - h = \frac{[PO_4^{\equiv}]}{\gamma_T C},$$

where γ_B, γ_S, γ_T are the activity coefficients of hydroxide, secondary and tertiary phosphates of sodium or potassium respectively and C is the total molar concentration of all phosphates. Substituting the values of OH^-, $HPO_4^=$ and $PO_4^{\equiv}$ from these relations in equation (2), we have

$$\frac{h^2 \gamma_B \gamma_S C^2}{(1-h)\gamma_T} = K_h \quad \ldots\ldots(4).$$

Also, the condition obtains that

$$[H^+][OH^-] = K_w.$$

Dividing this by equation (2),

$$\frac{[H^+][PO_4^{\equiv}]}{[HPO_4^=]} = \frac{K_w}{K_h} = K_a \quad \ldots\ldots(5),$$

where K_a is the tertiary ion activity or true constant.

The apparent dissociation constant may also be expressed in terms of the degree of hydrolysis of the tertiary salt

$$K_3' = \frac{[H^+][Na_3PO_4]}{Na_2HPO_4} \quad \ldots\ldots(6).$$

Replacing for the secondary and tertiary salt concentrations their equivalents from the above relationships

$$K_3' = [H^+]\left(\frac{1-h}{h}\right) \qquad \text{......(7).}$$

Expressed in logarithmic form

$$p_H = p_{K_a'} + \log\frac{1-h}{h} \qquad \text{......(8).}$$

Experimental.

Method of procedure. Purely prepared sodium and potassium phosphates were made up to definite concentrations with conductivity water at 20° and kept in pyrex flasks. The hydrion concentrations of these solutions were measured potentiometrically in a thermostat maintained at 20° and 38° ± 0·02° respectively. The gas chain consisted of Pt—H_2—solution x—saturated KCl—HgCl—Hg. The hydrion potentials measured for these solutions at varying time intervals showed that the hydrolytic equilibria are established immediately on preparation of the solution.

The hydrion concentration measurements were standardised daily against 0·1 N HCl prepared from constant boiling acid. The p_H was 1·085 at 20° and 1·09 at 38°, extrapolated from the activity coefficients determined by Noyes and Ellis [1917]. The calculated E.M.F. values of the entire system against the acid standard were 0·3119 at 20° and 0·3022 volt at 38°. The average divergence in terms of p_H was 0·003. The observed voltages were corrected to one atmosphere of hydrogen.

Preparation and analysis of materials. The tertiary phosphates were recrystallised twice from distilled water free from CO_2 and finally again by the addition of an equal volume of 95 % alcohol and then cooling in ice water. Two liquid phases were formed upon addition of alcohol, crystallisation proceeded at the interface while the upper phase gradually disappeared. The upper layer in the closed system prevented the absorption of CO_2 which otherwise would have tended to form the secondary phosphate and carbonate. The $Na_3PO_4 . 12H_2O$ crystals are trigonal and the $K_3PO_4 . 2H_2O$ are needle-like. The crystals were filtered on a Büchner funnel with suction and washed several times with alcohol. They were dried on a glass plate and finally pulverised. No attempt was made to prepare constant hydrates but the crystals were dried to remove free moisture and analysed for water of hydration. The water content was determined by first weighing a sample of the material in a platinum crucible and then heating in the electric furnace until constant weight was obtained.

The dodecahydrated trisodium phosphate crystals were tested by determining the transition temperature, 73·4°, which Richards proposed as a fixed point in thermometry. Analysis of the phosphates gave the following results.

Percentage composition of the phosphates.

Salt	Water determined	P Determined	P Calculated
K_2HPO_4	27·41	13·82	13·86
Na_2HPO_4	45·65	12·70	12·77
K_3PO_4	34·84	9·50	9·47
Na_3PO_4	44·74	10·45	10·49

Experimental and calculated data.

$[H^+]$ is the activity of the hydrion taken as that determined by the hydrogen electrode.

γ_B, γ_S and γ_T are the activity coefficients of the hydroxide, secondary and tertiary phosphates, respectively of sodium or potassium. They are the thermodynamic degrees of dissociation obtained by dividing the mean activity of the ions or their effective concentrations by the molarity of the salt. The coefficients given were read from curves plotted from Lewis and Randall's data [1923] for uni-, bi- and ter-valent anions at the calculated ionic strengths. The validity of this procedure follows especially from recent work which indicates that the ratio of the activity coefficient of an ion of one valency to that of another is constant at a given ionic strength.

Table I. *The tertiary dissociation constant of phosphoric acid.*

Calculated from the hydrolysis of Na_3PO_4 at 20°.

C	H^+	OH^-	μ	γ_B	γ_S	γ_T	h	K_h	$K_a \cdot 10^{12}$	$K_a' \cdot 10^{-12}$
0·0250	$0·907 \times 10^{-12}$	$9·48 \times 10^{-3}$	0·126	0·79	0·23	0·18	0·480	$1·12 \times 10^{-2}$	0·77	0·98
0·0125	1·418 ,,	6·03 ,,	0·065	0·84	0·33	0·26	0·574	1·03 ,,	0·83	1·06
0·00625	2·263 ,,	3·80 ,,	0·029	0·88	0·42	0·33	0·691	1·08 ,,	0·80	1·01
0·00312	3·806 ,,	2·26 ,,	0·014	0·90	0·51	0·41	0·805	1·16 ,,	0·74	0·92

Calculated from the hydrolysis of K_3PO_4 at 20°.

C	H^+	OH^-	μ	γ_B	γ_S	γ_T	h	K_h	$K_a \cdot 10^{12}$	$K_a' \cdot 10^{-12}$
0·0250	$0·930 \times 10^{-12}$	$9·25 \times 10^{-3}$	0·124	0·80	0·24	0·19	0·468	$1·04 \times 10^{-2}$	0·83	1·06
0·0125	1·431 ,,	6·01 ,,	0·065	0·84	0·33	0·26	0·572	1·02 ,,	0·84	1·07
0·00625	2·278 ,,	3·77 ,,	0·029	0·88	0·43	0·33	0·685	1·04 ,,	0·82	1·04
0·00312	3·858 ,,	2·23 ,,	0·015	0·91	0·51	0·41	0·784	1·00 ,,	0·85	1·04

Calculated from the hydrolysis of Na_3PO_4 at 38°.

C	H^+	OH^-	μ	γ_B	γ_S	γ_T	h	K_h	$K_a \cdot 10^{12}$	$K_a' \cdot 10^{-12}$
0·0250	$2·591 \times 10^{-12}$	$1·29 \times 10^{-2}$	0·115	0·80	0·24	0·19	0·648	$3·02 \times 10^{-2}$	1·11	1·40
0·0125	4·203 ,,	0·797 ,,	0·056	0·84	0·33	0·27	0·759	3·07 ,,	1·09	1·33
0·00625	7·202 ,,	0·465 ,,	0·027	0·88	0·43	0·34	0·846	3·23 ,,	1·03	1·31
0·00312	1·305 ,,	0·257 ,,	0·013	0·91	0·52	0·43	0·914	3·33 ,,	1·00	1·22

Calculated from the hydrolysis of K_3PO_4 at 38°.

C	H^+	OH^-	μ	γ_B	γ_S	γ_T	h	K_h	$K_a \cdot 10^{12}$	$K_a' \cdot 10^{-12}$
0·0250	$2·681 \times 10^{-12}$	$1·26 \times 10^{-2}$	0·115	0·80	0·24	0·19	0·629	$2·70 \times 10^{-2}$	1·24	1·57
0·0125	4·312 ,,	0·77 ,,	0·056	0·84	0·33	0·27	0·740	2·71 ,,	1·24	1·52
0·00625	7·261 ,,	0·46 ,,	0·027	0·88	0·43	0·34	0·839	3·01 ,,	1·12	1·41
0·00312	1·332 ,,	0·25 ,,	0·013	0·91	0·52	0·43	0·887	2·39 ,,	1·39	1·68

Table II. *The tertiary dissociation constant of phosphoric acid.*

Calculated from K_2HPO_4—K_3PO_4 mixtures

$$K_3 = \frac{[H^+]\left[B - \frac{[OH^-]}{\gamma_B} + \frac{[H^+]}{\gamma_S}\right]}{C - B + \frac{[OH^-]}{\gamma_B} - [H^+]}.$$

C	B	p_H	H^+	OH^-	μ	γ_B	γ_S	γ_t	$K_a'.10^{-12}$
0·0313	0·0062	11·20	6·31 × 100⁻²	0·0016	0·11	0·80	0·25	0·20	1·06
0·0294	0·0118	11·55	2·82 ,,	0·0036	0·15	0·76	0·21	0·16	1·10
0·0278	0·0168	11·80	1·59 ,,	0·0063	0·12	0·78	0·23	0·19	0·94
0·0263	0·0211	11·95	1·12 ,,	0·0089	0·12	0·79	0·23	0·19	0·84

μ is the total ionic strength. The ionic strength is equal to the stoichiometric molarity of each ion multiplied by the square of its valence. The total ionic strength is equal to the sum of the strengths of the anions and cations divided by two. The activity coefficient of a strong electrolyte is the same in all dilute solutions of the same ionic strength.

K_h is calculated from equation (4), K_a from equation (5), and K_a' from equations (6), (7) or (8). K_w at 20° is $0·86 \times 10^{-14}$ and at 38° is $3·35 \times 10^{-14}$.

Discussion of K_3 calculated.

The tertiary dissociation constant may be calculated from the data obtained by the electrometric titration of phosphoric acid and from the formulae developed by Van Slyke [1922]. The equation for the dissociation of a weak acid in an alkaline system is No. 29 of Van Slyke's paper,

$$K_a' = \frac{[H^+]\left(B - \frac{[OH^-]}{\gamma_B} + \frac{[H^+]}{\gamma_S}\right)}{C - B + \frac{[OH^-]}{\gamma_B} - [H^+]}.$$

Table III of that paper gives K_a' calculated from the titration data of W. M. Clark for $H(K_2PO_4) + KOH$. The above equation was used in its simplified form assuming complete dissociation. A recalculation of K_2' using activity coefficients at the respective ionic strengths is given in Table III. These values are concordant with those obtained from the hydrolysis data.

The apparent tertiary constant may also be calculated from the apparent secondary dissociation constant knowing the hydrion concentration which defines the secondary phosphate. Replacing the secondary and tertiary salt concentrations in equation (6) by their equivalents,

$$[Na_3PO_4] = \frac{K_3'}{K_3' + [H^+]} \cdot [C],$$

$$[Na_2HPO_4] = \frac{K_2'}{K_2' + [H^+]} \cdot [C]$$

and solving the quadratic, we obtain,

$$K_3' = \frac{[H^+]^2}{K_2'}.$$

Therefore, the secondary phosphate ratio has a maximum when $[H^+]$ is the geometric mean of the apparent dissociation constants,

$$p_H = \frac{p_{K_2'} + p_{K_3'}}{2}.$$

The p_H of the secondary phosphate is difficult to localise experimentally. Clark's titration curve gives p_H 9·3 for 0·066 M K_2HPO_4, $p_{K_2} = 6·8$ and hence p_{K_3} is 11·8. The secondary phosphates prepared in this work had a p_H of 9·35 and hence p_{K_3} is 11·9.

Table III. *The tertiary dissociation constant of phosphoric acid.*

Calculated from titration data of W. M. Clark for $K_2HPO_4 + KOH$.

C	B	p_H	H	OH^-	U	γ_B	γ_S	γ_T	$K_a'.10^{-12}$
0·0313	0·0062	11·20	$6·31 \times 100^{-2}$	0·0016	0·11	0·80	0·25	0·20	1·06
0·0294	0·0118	11·55	2·82 ,,	0·0036	0·15	0·76	0·21	0·16	1·10
0·0278	0·0168	11·80	1·59 ,,	0·0063	0·12	0·78	0·23	0·19	0·94
0·0263	0·0211	11·95	1·12 ,,	0·0089	0·12	0·79	0·23	0·19	0·84

Conclusion.

The tertiary dissociation constant of phosphoric acid, determined from the hydrolysis of the tertiary phosphates, has been found to be $1·02 \times 10^{-12}$ at 20° and $1·48 \times 10^{-12}$ at 38°.

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LXVIII. THE CATALYTIC ACTION OF CUPRIC SALTS IN PROMOTING THE OXIDATION OF FATTY ACIDS BY HYDROGEN PEROXIDE.

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During the last few years experiments on the oxidation of the higher fatty acids by means of hydrogen peroxide in the presence of ferrous iron have been carried out in this laboratory by C. G. Daubney; the results however were found to be exceedingly irregular. Under conditions which were apparently similar, in some experiments oleic acid was rapidly attacked and largely broken down to carbonic, formic, acetic and succinic acids; in other experiments hardly any action took place. The alteration in the conditions, however, which so greatly increased the oxidation eluded discovery. In a further attempt to solve this problem, the present authors endeavoured to investigate the effect of the addition of small quantities of impurities to the reaction mixture.

The conditions of the original experiments were as follows. Into a large flask were introduced 2·5 g. oleic acid, 10·6 cc. N sodium or ammonium hydroxide and 125 cc. distilled water: when the oleic acid was dissolved, 15 cc. of a solution containing 0·613 g. ammonium ferrous sulphate and 350 cc. hydrogen peroxide solution (20 vols.) were added to the soap solution. The flask fitted with a reflux condenser was left for 2 hours in a bath at 60°, the outlet being connected to an apparatus for the absorption of CO_2. At the end of this time, 2 cc. concentrated sulphuric acid were added and nitrogen was blown through the apparatus for a further 2 hours. Any insoluble acid was then filtered off and the liquid steam-distilled.

We first investigated the effect of the addition of small amounts respectively of nickel, manganese, zinc and copper salts which were added to the mixture at the same time as the ferrous salts. It was at once evident that the reaction was influenced by the addition of the copper salt but no certain effect was noticeable when nickel, zinc or manganese salt was added. Next we tried the effect of replacing the ferrous salt by an equivalent amount of cupric salt and we found that consistent results were obtained. The oxidation was very much more marked than in the "inactive" experiments with the ferrous salt, but the proportion of volatile acid was never quite as great as in the "active" iron experiments. Since we had now found conditions which were always repeatable, and since under the influence of the cupric salt at 60° in the presence of excess of hydrogen peroxide oleic acid may be largely

broken down into carbonic, formic, acetic and succinic acids, we decided to investigate this reaction quantitatively. We do not, however, think that the condition in the original experiments with ferrous salts which determined their activity was the presence of copper salts.

The influence of copper salts in promoting oxidation.

A study of the literature showed that the action of cupric salts in promoting the oxidation of tincture of guaiacum in the presence of hydrogen peroxide had been frequently noted and that one or two other instances of their action had been observed. Bourquelot and Bougault [1897] first recorded that in the presence of copper salts tincture of guaiacum is coloured blue, just as it is by the action of oxidising ferments. Here the oxygen necessary for the oxidation is derived from the air, and the copper salt behaves as an oxidase. Thus, if to a 2 % solution of quinone two or three drops of a 1 % solution of copper acetate are added, the characteristic blue colour is produced slowly in the cold, more rapidly on heating. The presence of oxygen is essential and the colour is not produced *in vacuo*. Labat [1908] recorded that an intense red colour was produced with the Kastle-Meyer reagent (an alkaline solution of phenolphthalein decolorised by boiling with zinc dust) in the presence of hydrogen peroxide to which a drop of a 2 % solution of copper sulphate in glycerol had been added. Mueller [1918] again confirmed the observations of Bourquelot and Bougault and drew attention to the behaviour of cupric salts both as oxidases, the supply of oxygen being obtained from the air, and as peroxidases, hydrogen peroxide acting as the source of the oxygen used in the reaction. Mueller suggested that the cupric salt reacts with the hydrogen peroxide, one oxygen atom of the peroxide entering into combination with the partial valencies of the copper and that this unstable compound then reacts with the oxidisable compound.

Thomas and Carpentier [1921] repeated Labat's observation and showed that the Kastle-Meyer reagent is extraordinarily sensitive to the action of copper salts, the colour being perceptible with a solution of copper containing only one part in a hundred million; indeed the distilled water found in most laboratories where copper stills are used for its preparation was found to be active. The close resemblance of this action to that of a peroxidase was again stressed by these authors, who also made the interesting observation that the lactate and tartrate of copper were inactive, but that the acetate and butyrate were active, behaving like the mineral salts. Aloy and Valdiguié [1923] extended these observations and brought forward evidence in support of the view that the intermediate substance concerned in this reaction is an unstable cupric peroxide: this substance they prepared by the method originally described by Thénard and also by the addition of a solution of hydrogen peroxide to one of ammoniacal copper sulphate, and they found that the peroxide behaved as a peroxidase. The activation of oxygen by metallic copper was studied by Wieland [1923]; in his experiments various organic

acids, acetic, pyruvic, succinic, malic, benzoic, oxalic and lactic, were oxidised with the production of carbonic acid, the acids being shaken with oxygen in the presence of metallic copper. He found that the reaction only took place in acid solution and that when the metal had dissolved in the acid oxidation was at an end. He found a similar effect when cuprous salts were added in the presence of hydrogen peroxide and here again the effect was only produced in acid solution and came to an end when the cuprous salt was converted to the cupric state. He says "Vor allem aber büsst das peroxydische oder höherwertige Kupfer nach Abgabe eines Sauerstoffaequivalentes als Cuprisalz sein Oxydationsvermögen ein."

With regard to this we can only say that under the conditions of our experiments the cupric salts are certainly the active agents. Since, as far as our investigations go, the presence of an acid group in the molecule seems essential in order that oxidation may take place, it seems to us possible that the copper enters into the molecule of the oxidisable acid, forming a salt, and that the partial valencies of the copper then unite with the oxygen of the hydrogen peroxide as Mueller suggested, forming an unstable intermediate compound.

The close analogy of the action of cupric salts with that of the peroxidases made us hopeful that the oxidation of the higher fatty acids by means of hydrogen peroxide in the presence of cupric salts might present a closer analogy with the physiological process than oxidation by any other known laboratory method. When the higher fatty acids are acted upon by hydrogen peroxide in the absence of a catalyst they are very little attacked and therefore it has been difficult to establish the nature of the reaction. We decided therefore first to work out the method on the simpler fatty acids and their oxygenated derivatives and later to apply the method to a comparison of the oxidation of palmitic, oleic and stearic acids.

Preliminary experiments on the oxidation of oleic acid indicated that among the products of the reaction were acetic and succinic acids. The behaviour of these acids under the same conditions of oxidation was therefore examined and the reaction extended to other acids which might conceivably occur as intermediate products. Succinic acid was chosen for the investigation of the conditions of the reaction.

Influence of the reaction of the medium. In alkaline or neutral solution very little oxidation of succinic acid occurs; favourable results were obtained when the acidity of the mixture corresponded to 0·010 to 0·020 *N*. It seems essential that the reaction shall be acid but small changes in the hydrogen ion concentration appear to exert little influence.

Method.

2·5 g. of the acid to be oxidised were placed in a large flask and the calculated quantity of *N* NaOH solution was added, in most cases sufficient to make the whole reaction mixture equivalent to an acidity of 0·015 to

0·020 *N*. A measured quantity of a 0·73 % solution of crystalline cupric sulphate and 350 cc. of a previously neutralised solution of hydrogen peroxide (20 vols.) were then added, the latter reagent being present therefore in large excess. The mixture was left in a bath at 60° for a stated time, the outlet of the flask being connected with an absorption apparatus containing a known amount of a standard baryta solution: 2 cc. concentrated sulphuric acid were added to the contents of the flask and nitrogen was blown through for 1½ hours, the bath being meanwhile allowed to cool. When the oxidisable acid is insoluble in water, the addition of the sulphuric acid precipitates any insoluble acid and so prevents the further action of the peroxide. In the case of soluble acids, however, the effect of the hydrogen peroxide on the acid continues though at a lower temperature and in a much more acid medium during the time in which the carbonic acid is being blown off.

Table I.

(*a*) during time of experiment;
(*b*) during subsequent steam-distillation at 100°.

Succinic acid g.	Time of exp. hr.	Reaction of mixture	Atomic proportion Cu to 1 mol. succinic acid	Percentage of original succinic acid appearing as: CO_2 (*a*)	CO_2 (*b*)	Formic	Acetic
Influence of reaction of medium:							
		Alkali					
2·5	2	0·054 *N*	0·000	4·0	—	—	—
,,	1	,,	0·077	0·6	—	—	—
,,	0·5	,,	0·077	0·8	—	—	—
		Acid					
,,	1	0·015 *N*	0·000	7·1	37 1	—	—
,,	1	,,	0·077	45·2	6·0	—	—
,,	0·5	,,	0·077	41·2	8·0	3·9	26·5
Influence of proportion of copper:							
		Acid					
2·5	0·5	0·012 *N*	0·000	Trace	—	—	—
,,	,,	0·015 *N*	0·026	9·4	—	—	—
,,	,,	0·018 *N*	0·052	28·8	15·0	5·0	22·0*
,,	,,	0·020 *N*	0·078	53·4	5·0	2·0	17·6*
Influence of time of experiment:							
		Acid					
2·5	0·5	0·014 *N*	0·026	9·7	—	—	—
,,	1	,,	,,	29·6	—	—	—
,,	1·5	,,	,,	48·9	—	—	—
,,	2	,,	,,	56·9	—	—	—

* In these experiments the hydrogen peroxide was removed before the steam-distillation.

The acid mixture is then filtered. In the earlier experiments the solution was then steam-distilled, but in this case the excess of hydrogen peroxide continues to act at a higher temperature on any products in the solution. In the later experiments, therefore, before carrying out the steam-distillation, the excess of hydrogen peroxide was destroyed by making the solution alkaline, adding to it finely powdered charcoal and allowing it to stand overnight.

The next morning the filtered solution was again acidified and steam-distilled, the distillate collected, any emergent gas being passed through a standard baryta solution. The distillate was neutralised with a known amount of N NaOH solution and evaporated to dryness. The amount of formate in the sodium salts of the volatile acids was estimated, and the molecular weight determined. In most cases the acid is completely soluble and appears to consist almost entirely of acetic and formic acids. Aldehyde was usually present and was estimated in an aliquot part of the original distillate. The results of the experiments with succinic acid which are collected in Table I show (1) that the addition of the cupric salt is effective in acid but not in alkaline solution; (2) that after the mixture has stood for half an hour at 60°, about 75 % of the succinic acid has been decomposed into carbon dioxide, formic acid and a notable proportion of acetic acid.

Comparison of the relative effect of ferrous, cupric and cuprous salts.

Neither in acid nor in alkaline solution did we find that ferrous salts influenced the oxidation of succinic acid under the conditions of our experiments. The amount of oxidation was here measured by the percentage of the original acid appearing as CO_2 during the time of the experiment. It will be seen that the results of the experiments in which iron was present were similar to those in which no metallic salt was added. The possibility that intermediate products of oxidation were present was not however excluded.

Table II. *Showing the comparative effect of cupric and ferrous salts on the oxidation of succinic acid by hydrogen peroxide.*

Succinic acid g.	Time of exp. hr.	Reaction of mixture (acid)	Metal added	Percentage of original suc acid conve to CO_2
2·5	1	0·022 N	—	7·2
,,	0·5	0·014 N	Fe	6·0
,,	0·5	,,	Fe	8·8
,,	1	,,	Fe	5·2
,,	1	,,	Fe	4·4
,,	0·5	,,	Cu	41·2
,,	1	,,	Cu	50·4
,,	1	,,	Cu	46·4
,,	1	,,	Cu	54·0
,,	1	,,	Cu	45·2

0·078 of an atomic proportion of Cu″ or Fe″ was taken in each case.

The substitution of cupric chloride or cuprous chloride for cupric sulphate gave similar results. The action of the cuprous chloride was rather slower than that of the cupric compound. The effect of the same salts in promoting the decomposition of the hydrogen peroxide was then studied, the decomposition being measured by the volume of oxygen evolved in the same time under similar conditions at 60°.

70 cc. hydrogen peroxide were neutralised with 1·4 cc. N NaOH, 23·6 cc. water and 2 cc. N acid added and equivalent amounts of cupric and

cuprous chloride and of ferrous sulphate respectively. The relative amounts of oxygen liberated in the same period of time when measured under similar conditions were respectively 910 cc. ($CuCl_2$), 610 cc. ($CuCl$) and 215 cc. ($FeSO_4$). The control in the absence of metallic salts liberated 160 cc.

It appears therefore that cuprous salts are more effective in bringing about the decomposition of hydrogen peroxide but that cupric salts are more active in catalysing the oxidation of the aliphatic acids by hydrogen peroxide.

We then investigated the effect of the same oxidising agent on some of the acids which might possibly occur as oxidation products of succinic acid and the results which we obtained are set forth in the following table (Table III). In each case the reaction of the mixture was 0·014 *N* acid and 0·078 of an atomic proportion of copper was present for each molecular proportion of the acid to be oxidised: the conditions of the experiments were similar to those already described. The action was estimated by the weight of carbonic acid evolved during the time of the experiment and during the subsequent period in which the carbonic acid is being blown off from the solution. It is possible that, in the absence of the cupric salts, oxidation products containing the same number of carbon atoms may have been produced. We did not however obtain any evidence of this.

Table III.

			H_2O_2 and Cu		H_2O_2 alone	
Acid	g.	Time of exp. hr.	Weight CO_2 g.	Acid converted to CO_2 %	Weight CO_2 g.	Acid converted to CO_2 %
Succinic	2·5	0·5	1·54	41·2	—	Trace
		1	1·68	45·2	—	7·1
			2·01	54·0	—	—
			1·58	42·4	0·04	1·5
			1·87	50·2	—	—
			1·65	44·4	0·006	0·02
Malic	1·0	1	0·77	58·8	0·03	2·3
Tartaric	2·5	0·5	>1·68	>57·3	CO_2 evolved too rapidly	
		0·75	>1·93	>65·8	for complete absorption	
	1·0	0·5	1·18	100	0·0	0·0
		1	1·18	100	0·0	0·0
Dihydroxymaleic	1·0	1	0·76	64·0	0·29	24·45
	,,	1·25	0·72	60·2	0·34	28·60
Pyruvic	1·0	1	0·90	60·00	0·31	21·00
	,,	—	0·85	56·67	—	—
Lactic	1·0	1	0·63	43·3	0·002	0·14
	,,	—	0·56	38·0	—	—
Malonic	1·0	1	0·02	1·8	—	—
	,,	1·25	0·48	38·5	—	—
	,,	2	0·43	34·6	0·01	0·8
Acetic	2·5	1	1·22	33·3	—	—
	1·0	1	0·47	32·3	0·007	0·4

When tartaric acid is oxidised by means of hydrogen peroxide in the presence of ferrous iron, dihydroxymaleic acid is the first product of oxidation [Fenton, 1894, 1896]. Since, however, in the presence of cupric salts, dihydroxy-

maleic acid is much less readily oxidised to carbon dioxide than is tartaric acid, the evidence available does not support the view that, in the presence of cupric salts, tartaric acid is first converted to dihydroxymaleic acid. Malonic acid showed a curious period of lag before action began in three of our experiments, during the first hour there was practically no evolution of carbonic acid, then in the next 15 minutes a rapid evolution of gas took place. The notable proportion of acetic acid formed during the oxidation of succinic acid was interesting, and the fact that acetic acid itself was also converted to carbonic acid, one-third of the amount taken being completely oxidised to carbonic acid in an hour, was also noteworthy.

In the absence of the cupric salt only two of the acids taken were oxidised by the action of the hydrogen peroxide under the conditions of our experiments. These were pyruvic and dihydroxymaleic acids, but the evolution of carbon dioxide is much greater in the presence of the copper salt.

Similar experiments were carried out using alcohol, acetone and glycol as the materials to be oxidised, but in no case was there any evidence of oxidising action taking place. The addition of dilute sulphuric acid to the mixture produced no effect, and the action of the cupric salt seems only effective when the substance to be oxidised is an acid and the experiment is carried out when a certain proportion of the free organic acid is present.

The oxidation of the higher fatty acids is at present under investigation, but as the method seems to be of general application for the oxidation of the aliphatic acids, we desire to record these preliminary results.

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LXIX. AN IMMEDIATE ACID CHANGE IN SHED BLOOD.

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Introduction.

The p_H of blood was measured at body temperature by the glass electrode method about 2 minutes after the withdrawal of the blood from the blood-vessel. The p_H was observed to decrease by about 0·05 a few minutes later. This acid change seemed to be very important, as it meant that later determinations do not represent the p_H of the blood in the body. A typical p_H-time curve is shown in Fig. 1, curve 1. This p_H change was investigated further.

Technique.

(*a*) *Collection of blood.*

Human blood was mainly used in this research, the total number of individuals from whom one or more blood samples were taken being 21. No special attempt was made to control the physiological conditions which might affect the c_H of the blood, but the subject sat still for 5–10 minutes before the blood-vessel was punctured. Stasis was generally avoided, but in the later experiments it was used when convenient.

The skin was cleaned with an antiseptic, the vessel punctured with a sterile needle, and the blood was drawn into a syringe. Contact of the blood with air was avoided by filling the dead space of the syringe and needle with liquid paraffin. Change of temperature during this manipulation was reduced to a minimum. This could be done by using a small all-glass syringe, previously warmed to 38°. When a syringe with metal plunger and cap was used it was necessary to warm it to about 45°, and to lag it with cloth, as otherwise the conductivity of the metal caused it to cool quickly.

In the experiments in which chemical substances in solution were added to the blood, these were measured into the syringe before the puncture. The

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presence of a glass bead proved essential to ensure complete mixing. No liquid paraffin was used in these cases (except as a piston lubricant). The needle was inserted into the vessel, and blood allowed to flow through it before the syringe was attached. In this way air was removed from the needle and there was no danger of traces of the chemical entering into the circulation.

(*b*) *Measurement of* p_H.

The hydrogen ion concentration of the blood was measured by the glass electrode method [Kerridge, 1926]. The blood was transferred immediately from the warm syringe to a standardised electrode, which was at body temperature, and the first c_H determination completed between one or two minutes after the blood left the vessel.

Two completely independent glass electrode apparatus were used, so that simultaneous p_H-time curves could be obtained from samples of blood either at two different temperatures, or with and without added substances. The calomel and glass electrodes of each apparatus were enclosed in a large air-bath with automatic temperature regulation and an efficient fan. The air-baths were lined with metal, which was connected to earth. When the door of an air-bath was opened, as for the introduction of the sample of blood, the fan was stopped, and an accessory heating lamp, situated near the door, was turned on. It was very important that this procedure should be completed as quickly as possible, and the fan turned on again as soon as the door was shut. The temperature remained constant within 0·5° under these circumstances. The glass electrodes were all made of Wood's glass of the formula recommended by Hughes [1928]. They were standardised daily against a solution of $M/20$ potassium hydrogen phthalate, the p_H of which was taken to be 3·97°, both at room temperature and at 37·5°. The standardisations were carried out at the same temperature as the subsequent experiment. From time to time, the electrodes were checked by determining the p_H of a dilute phosphate buffer solution, of about p_H 7·3, which was also estimated by the quinhydrone electrode method. The calomel electrodes contained 3·5 M KCl, and the potassium phosphate buffer used on one side of the glass membrane was dilute (approx. $M/20$). Under these circumstances the liquid-liquid junction potentials can be neglected, but evidence was obtained which showed that this is by no means the case when the calomel electrodes contain $M/10$ KCl and the phosphate buffer solution is concentrated. Special care was taken with the ground caps of the calomel electrodes, as KCl may diffuse into the electrode during long experiments when these fit badly.

The Lindemann electrometers were adjusted so that a deflection caused by 0·25 mv. (corresponding to 0·004 p_H) could be detected. By the kind permission and courteous cooperation of the Cambridge Instrument Co., both potentiometers were calibrated at their works for zero error and uniformity of slide wire against one of their standard potentiometers. The potentiometers

were standardised before each experiment with a Weston cadmium cell. The error in the potentiometer readings is estimated at 0·5 %. Control experiments in which the p_H of the same liquid was measured in the two apparatus agreed within 0·02 p_H. This difference gives the order of the combined instrumental and manipulative errors. The control experiments were done both on buffer solutions, whose p_H was checked by the quinhydrone electrode method, and on blood. Thus it may be said that the absolute p_H measurements were accurate to 0·02 p_H, but that differences of 0·005 p_H on any one sample were significant.

Experiments.

(i) *Effect of sodium fluoride.*

Evans [1922] has described changes in blood shortly after shedding, which were stopped by sodium fluoride. We found that addition of 0·02 % sodium fluoride to the blood caused an almost complete disappearance of the change in p_H corresponding to the part of the p_H-time curve denoted at *DE* in Fig. 1. The inhibition was complete in greater concentrations, and we used 0·06 % in subsequent experiments in which we wished to eliminate this p_H change (cf. Fig. 1, curve 2).

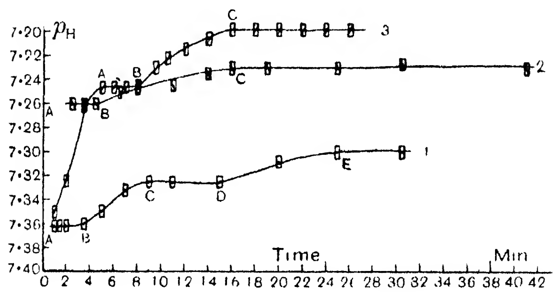

Fig. 1. p_H-time curves of shed blood.

Curve 1. Venous blood (human).

Curve 2. Venous blood (human) + 0·06 % sodium fluoride.

Curve 3. Arterial blood (cat under chloroform) + 0·04 % potassium oxalate + 0·06 % sodium fluoride: kept at 0° until 1 min. before experiment.

The abscissae represent time in minutes after the blood left the vessel in curves 1 and 2, and after the blood was put in the electrode in curve 3. The rectangles represent size of experimental errors.

AB corresponds to "latent period."

BC corresponds to "first acid change."

DE corresponds to acid change inhibited by sodium fluoride.

We propose to use in the remainder of this paper the term "first acid change" for the change in p_H corresponding to the part of the curve in Fig. 1 labelled *BC*. It is this change which has occupied most of our attention. The time before the beginning of the first acid change, labelled *AB*, will be referred to as the "latent period."

(ii) *The "first acid change."*

Particulars of p_H-time curves at 38° and 28° are given in Table I. Both the latent period and the duration were greater at the lower temperature, although they varied between rather wide limits. No figure has been given for the latent period of venous blood at 38° as in 12 out of 31 experiments the "first acid change" had apparently commenced before the first p_H reading was taken about 2 minutes after shedding; of the remainder, 10 latent periods were between 2 and 8 minutes, and the others had widely different values up to a maximum of 40 minutes.

A decrease in p_H of 0·05 corresponds to a 12·5 % increase in hydrogen ion concentration, and an average deviation from the mean of 0·015 p_H, to a 3·5 % variation. The variation is of the same order when only repeated determinations on the blood of a single individual are considered.

There were no significant differences between the results on arterial and on venous blood.

Table I.

	Venous blood		Venous blood + 0·04 % K oxal. + 0·06 % NaF		Arterial blood
Temperature	38°	28°	38°	28°	38°
Latent period (min.):					
Mean	—	11	6	19	7·5
Maximum	40	13	14	30	17
Minimum	1·5	8	3	12	2·5
First acid change (p_H):					
Mean	0·055	0·05	0·04	0·05	0·05
Maximum	0·095	0·06	0·08	0·09	0·08
Minimum	0·02	0·03	0·015	0·03	0·025
Average deviation from mean	0·015	—	0·01	0·02	0·015
No. of experiments	31	3	9	7	7
Duration of first acid change (min.):					
Mean	9	5	10	18	5
Maximum	21	9	15	30	9
Minimum	2	2·5	1	2	2·5

At room temperature (19·5° ± 1·5°) there was no significant change in p_H, greater than the experimental error, within about $1\frac{1}{2}$ hours after the blood was shed, but then an acid change took place very slowly, after which the p_H again became constant.

(iii) *Effect of anticoagulants.*

In the course of the experiments already described the blood clotted in the electrode. The following anticoagulants were added to the blood, without affecting the "first acid change":

Heparin 0·02 %, 0·04 % and 0·08 %; sodium fluoride 0·3 %; ammonium oxalate 0·045 %; potassium oxalate 0·04 %.

The substances, dissolved in isotonic saline, were added to the blood in such concentration as to give a 10 % dilution of the blood. Control experiments,

in which dilution was made with isotonic saline alone, showed no measurable change in the initial p_H of the blood or in the "first acid change."

Heparin (0·02, 0·04 or 0·08 %) and sodium fluoride (0·06 %) added to the blood together do not inhibit the acid change corresponding to the part of the curve in Fig. 1 labelled *DE*, which is inhibited by sodium fluoride alone. We have no explanation to offer.

Data referring to the "first acid change" in the presence of potassium oxalate (0·04 %) and sodium fluoride (0·06 %) are given in Table 1, columns 3 and 4. It will be noted that the mean latent period is greater than in the case of blood alone, and that the mean size of the "first acid change" is less, although the difference in dimension is of questionable significance.

(iv) *Effect of potassium cyanide and of thymol.*

Potassium cyanide was added in $M/100$ concentration, without affecting the shape of the p_H-time curve. The cyanide made the initial p_H more alkaline, unless it was previously neutralised. Thymol ($M/500$) likewise had no effect.

(v) p_H*-time curves of plasma.*

The latent period of the "first acid change" of venous blood containing potassium oxalate (0·04 %) and sodium fluoride (0·06 %) had been found to have a mean value of 19 minutes at 28°. In another series of experiments the blood was centrifuged at 28° during this latent period, and the plasma was separated and placed in the electrode. In a typical experiment centrifuging was carried out between the fourth and eighth minutes, and the first p_H measurements were made at the tenth minute after the blood was shed. The plasma p_H-time curves showed no "first acid change." Corpuscles with adherent plasma showed typical p_H-time curves with a "first acid change." Plasma to which corpuscles were added to give one-quarter of the normal number showed curves with a typical "first acid change."

(vi) *Effect of laking the blood.*

p_H-time curves on laked blood were made from venous blood containing potassium oxalate (0·04 %), sodium fluoride (0·06 %) and saponin (1 %). These showed typical "first acid changes." The same result was obtained when laked blood was centrifuged at room temperature, before the experiment, to free the fluid from white cells.

(vii) *Miscellaneous experiments.*

Any possible unexplained electrical effects of the fibrin or of the corpuscles settling on to the glass membrane were ruled out as the cause of the "first acid change" by reversing the customary arrangement of buffer and blood in the electrode. The "first acid change" still occurred when the corpuscles dropped away from the membrane.

Two samples of blood with potassium oxalate and sodium fluoride kept at 38° for 0;5 hour, and for 1 hour respectively, before transference to the

glass electrode, showed no change of p_H with time. Their p_H agreed with the final p_H of another sample of blood which had been placed in the electrode as soon as possible after it was shed, and which had undergone a typical "first acid change" in the electrode. Hence in the first two cases the "first acid change" may be presumed to have taken place while the blood was still in the syringe, and to be quite independent of the electrode. Similarly blood containing heparin and hydroxyquinoline potassium sulphate (as an antiseptic) showed no change of p_H with time after 22·5 hours at 38°.

Estimations were made of the total carbon dioxide and of the oxygen contents of blood before and after the "first acid change". In these experiments a sample of blood was drawn into a syringe at 0°, which contained potassium oxalate and sodium fluoride. One portion was taken immediately for analysis. The remainder was warmed to 38° and kept at that temperature for 0·5 hour before an analysis was made. The method of analysis used was that of Harington and Van Slyke [1924]. No appreciable difference between the O_2 and total CO_2 contents of the two samples could be detected.

Samples of blood were taken in a similar way for lactic acid estimations. These were kindly made for us by Dr H. D. Kay by the method of Friedemann, Cotonio and Shaffer [1927], on the ether extract of a trichloroacetic acid filtrate of the blood. No significant difference between the results on the two samples was observed. However, it is possible that the "first acid change" might be occasioned in the first sample in the course of the precipitation with trichloroacetic acid, and it is therefore impossible to pronounce definitely that the acid which causes the shift in hydrogen ion concentration is not lactic acid.

Discussion.

The results of the experiments described in the previous section may be summarised as follows. The "first acid change" is not associated with the clotting of blood; it is delayed by decrease of temperature; it is not affected by substances which inhibit oxidation phenomena (KCN), enzyme action (thymol), or glycolysis (NaF). It does not occur in plasma, but does occur when the red corpuscle wall is broken down and in the absence of leucocytes. No explanation has been found.

There are references in the literature to changes in p_H accompanying clotting. Kugelmass [1922] reported an alkaline change of 0·3 p_H, using a hydrogen electrode on a solution of diluted oxalated plasma. Hirsch [1924] found an acid change of 0·09 p_H in rabbit's plasma by a colorimetric method. Ross [1921] found no change in the p_H of blood on clotting. Our experiments confirm those of Ross, as the p_H-time curves of blood at 38° and 28° were unaffected by the addition of anticoagulants, and as in the experiments at room temperature the blood clotted in the electrode, while the p_H remained constant.

The existence of this unexplained "first acid change" signifies that p_H measurements of blood at 38° made more than about 4 minutes after the

blood is shed will be more acid than when it leaves the blood-vessel, by an average of 0·05 p_H. This appears to be due to an increase in acidity within the red corpuscle, as it occurs in centrifuged laked blood, but not in plasma. From the buffer curves of blood of Van Slyke, Hastings, and Neill [1922], it can be calculated that an increase in acidity of 0·05 p_H corresponds to an addition of 1·2 millimol of acid per litre of blood. Assuming that this p_H change occurs at constant CO_2 content, it must be accompanied by a disturbance of the $\frac{[H_2CO_3]}{[BHCO_3]}$ ratio in the blood. The corresponding changes in $[H_2CO_3]$ and $[BHCO_3]$ can be calculated by the Henderson-Hasselbalch equation. At 38°, assuming $pK = 6·17$ and constant, the change in $[H_2CO_3]$ is 11 %, in $[BHCO_3]$ 5 %. Thus, blood having initial pCO_2 of 48 mm., and combined CO_2 of 45·5 vols. %, would have 53 mm. and 43·3 vols. % respectively after the "first acid change." Thus, delayed determinations of pCO_2 and vCO_2 of blood will misrepresent the amounts at the time of shedding by this amount. At 28°, as the CO_2 tension is lower, the difference in pCO_2 comes within the experimental error of the methods available, thus thwarting an attempt to demonstrate independently the "first acid change" by measuring the CO_2 tension of blood during the latent period, and after the acid change had taken place. A "first acid change" has been also observed in the blood of cats, dogs and rabbits. Experiments on the behaviour of blood in pathological conditions are in progress.

Precautions for measurement of p_H of blood.

There remains to be considered the best practical method of measuring the p_H of blood as it leaves the blood-vessel. It may be estimated at 38° within 1 or 2 minutes, with care taken to avoid cooling in transference from vessel to electrode. This, however, is not always convenient. Secondly, potassium oxalate (0·04 %) and sodium fluoride (0·06 %) may be added, the determination made less hurriedly and a correction of 0·05 p_H applied for the "first acid change." The average variation from the mean correction in normals is only ± 0·015 p_H, but nevertheless the method is objectionable as we have no evidence as to the size of the correction, or as to its variation in different physiological conditions, or in pathological states. A third method is to measure the p_H at room temperature, when no acid change occurs up to 1·5–2 hours after the blood is shed, and to apply a temperature correction. We have found the mean p_H of venous blood to be 7·32 at 38°, 7·47 at 28°, and 7·60 at 19·5°. These correspond to a change in p_H of 0·015 per degree. Martin and Lepper [1926] give 0·011 p_H per degree. A correction of this magnitude introduces a considerable degree of uncertainty, especially as nothing is known of its variation in different physiological and pathological conditions of the blood. A fourth method is as follows.

The blood is rapidly cooled by drawing into an ice-cold syringe, and is kept in the syringe on ice until convenient. (Care must be taken that the

temperature is not very much below 0°, or the blood will freeze, and lake on thawing.) The blood is brought to room temperature immediately before transference to an electrode at 38°, and a p_H-time curve taken. A typical form is shown in Fig. 1, curve 3. The first part shows a decrease of p_H with temperature which corresponds to the change in p_H incident to the warming of the blood. This is complete in about 4–5 minutes, and is followed by a steady p_H period. The "first acid change," which is apparently delayed by the cooling, then follows. The "plateau" lasts about 5 minutes, and has been shown to agree with the p_H of a sample of the same blood measured immediately after it was shed. In one experiment the blood was left for as long as 17 hours on ice before measurement. This last method is in our opinion the best, when the p_H cannot be measured at 38° immediately. It does not require the introduction of any correction. and can be done at any convenient time subsequent to the puncture. The addition of oxalate and fluoride is not essential.

Summary.

1. An increase in the hydrogen ion concentration of blood has been found to take place soon after the blood is shed.

2. This change has a mean value of – 0·05 p_H at 38° and at 28°. It takes place within about 6 minutes at 38°, and 11 minutes at 28°. It does not occur until after 1·5–2 hours at room temperature.

3. The change is unaffected by sodium fluoride, anticoagulants, potassium cyanide or thymol. It takes place in laked blood and centrifuged laked blood but not in plasma.

4. It is suggested that blood should be cooled to 0° before subsequent p_H measurement at 38°. In this way the acid change can be delayed long enough to enable a satisfactory p_H estimation to be made.

We wish to thank Prof. J. Barcroft, Prof. Arthur Ellis, and Prof. C. Lovatt Evans for much helpful interest and criticism, Miss N. Henderson for assistance in the early part of the work, the Medical Research Council and the London Hospital Medical College Research Fund for defraying the expenses of the research, and the Cambridge Instrument Co. for the loan of instruments.

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LXX. STUDIES IN CARBOHYDRATE METABOLISM.

VI. THE ANTAGONISTIC ACTION OF PITUITRIN AND ADRENALINE UPON CARBOHYDRATE METABOLISM WITH SPECIAL REFERENCE TO THE GASEOUS EXCHANGE, THE INORGANIC BLOOD-PHOSPHATE AND THE BLOOD-SUGAR.

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In recent years a considerable amount of attention has been directed to the question of the physiological antagonism between pituitrin and insulin, particularly in relationship to carbohydrate metabolism.

With the discovery of insulin opportunities were afforded for studying more directly the interaction of these hormones, and interest in the subject was stimulated afresh by the observations of Burn [1923], who demonstrated that pituitrin could inhibit the fall in blood-sugar following administration of insulin and bring about recovery from insulin hypoglycaemia in rabbits. These results were confirmed by numerous observers [Olmstead and Logan, 1923; Moehlig and Ainslee, 1925; Lawrence and Hewlett, 1925; Voegtlin, Thompson and Dunn, 1925; Geiling and Britton, 1927]. The suggestion put forward by Burn that these findings might be explained by a direct and specific antagonism between pituitrin and insulin has resulted in attempts being made by other workers to look for evidence of this antagonism in other directions. Thus Joachimoglu and Metz [1924] have employed the isolated uterus, but the evidence of antagonism was not conclusive. Serebrijski and Vollmer [1925] and Koref and Mautner [1926] found that when insulin was injected simultaneously with pituitrin it did not inhibit the antidiuretic effect of the latter, but during insulin hypoglycaemia the antidiuretic action was abolished. Given separately both insulin and pituitrin were antidiuretic, insulin causing water retention in the tissues, and pituitrin acting directly upon the kidneys. The peculiar and variable effect of insulin upon blood concentration, producing hydraemia or anhydraemia according to the conditions, is a factor which will have to be taken into consideration in

interpreting these results [Drabkin and Edwards, 1924; Levine and Kolars, 1924–1925; Villa, 1924; Olmstead and Logan, 1923; Haldane, Kay and Smith, 1924; Olmstead and Taylor, 1924].

Coope and Chamberlain have shown that pituitrin and insulin are antagonistic as regards certain changes in fat metabolism [Coope, 1925; Coope and Chamberlain, 1925].

The facts so far accumulated show that certain metabolic processes are influenced in opposite directions by insulin and pituitrin, and to this extent they may be said to be mutually antagonistic, but conclusive evidence of a direct inactivation of one hormone by the other has not been obtained. The changes which occur in fat metabolism and water metabolism under insulin may merely be secondary results of the action of insulin upon the combustion and storage of carbohydrate. With regard to carbohydrate metabolism, it may be noted that investigations regarding the modification of insulin action by pituitrin have been almost entirely confined to observations upon the blood-sugar or upon symptoms dependent upon changes in blood-sugar concentration, and the same question arises as to whether the fact that the blood-sugar concentration is altered in the opposite direction by insulin and pituitrin is to be explained by direct inactivation or by the result of some secondary effects of the hormone. In this connection it may be mentioned that Lambie [1926] and Clark [1928] have found that in the decerebrate cat, with the liver excluded from the circulation and constant transfusion of dextrose, pituitrin is unable to prevent the fall in blood-sugar caused by insulin, and this would suggest that, in the intact animal, either the liver is necessary to effect the mutual inactivation of insulin and pituitrin, or it is merely the source of dextrose yielded up under the influence of pituitrin to maintain the blood-sugar at or above the normal level in experiments in which the organ is intact. In so far, however, as changes in the concentration of the blood-sugar are not necessarily a measure of utilisation of sugar in the tissues, it cannot be inferred with certainty that even with the liver excluded from the circulation pituitrin is without influence upon carbohydrate metabolism.

In order, if possible, to throw light upon the effect of pituitrin on the metabolism of carbohydrate, it was thought desirable to study the respiratory metabolism and the inorganic blood-phosphate after administration of carbohydrate or insulin together with pituitrin, and the experiments described below were undertaken with that object.

If pituitrin inactivates insulin, changes of a similar character to those met with in diabetes might be looked for.

Hines, Leese and Boyd [1927], in experiments upon dogs with continuous transfusion of dextrose and pituitrin, came to the conclusion that while pituitrin caused less sugar to be retained than in controls, it was without effect upon the respiratory quotient and heat production: but the conditions of these experiments may not have been favourable for obtaining a positive result.

In view of the alleged capacity of pituitrin to annul the action of insulin upon carbohydrate metabolism, it was thought that an opportunity might also be afforded to study the metabolism of dihydroxyacetone under these conditions and thus gain further information upon the question as to whether this triose can be utilised even when the metabolism of dextrose is inhibited.

Since adrenaline is another hormone which, like pituitrin, has an action opposite to that of insulin, in so far as it can raise the blood-sugar and cause recovery from insulin hypoglycaemia, it was deemed of interest to carry out some experiments in order to determine how the changes in the respiratory metabolism compared with those obtained with pituitrin.

EXPERIMENTAL.

A. Respiratory exchange, blood sugar, and inorganic blood-phosphate in the human subject.

As in previous experiments [Lambie and Redhead, 1927] the subject was in the post-absorptive condition. The expired air was collected in 6-minute samples, using the Douglas bag method, and was analysed by means of the Haldane apparatus. The basal metabolic rate and fasting blood-sugar were first determined, after which the substance to be investigated was administered, and subsequently samples of expired air and blood were taken at intervals. The blood-sugar was estimated by the Hagedorn-Jensen method. When intravenous administration was employed, the sugar (20 g.) was dissolved in 100 cc. of saline and sterilised by filtering through a Seitz germicide (EK) filter No. 6 using pads impermeable to organisms. The injection was made at a uniform rate and occupied a period of exactly 7 minutes.

Exp. 1 (*a*). Respiratory metabolism and blood-sugar curve in normal student after intravenous injection of 20 g. of dextrose.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.
0	+ 7	1·62	0·99	57·25	0·086
1–7	Dextrose intravenously				
1–3	+28	2·00	0·87	52·0	—
3–6	+ 6	1·72	0·73	45·0	—
10	+ 4	1·63	0·85	41·25	0·133
20	+11	1·73	0·89	44·0	0·108
30	+17	1·86	0·81	45·0	0·100
40	+ 8	1·72	0·82	44·75	0·087

Exp. 1 (*b*). Same as preceding + 0·75 cc. of pituitrin* administered subcutaneously while dextrose transfusion was proceeding. (Dextrose dissolved in 200 cc. saline.)

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.
0	+ 3	1·56	0·88	76	0·079
1–7	Dextrose intravenously + pituitrin subcutaneously				
10	+17	1·81	0·79	71	0·166
25	+ 4	1·56	0·91	71	0·146
40	+ 7	1·62	0·90	70·71	0·139
50	+18	1·80	0·87	77·65	0·070
80	+15	1·72	0·95	77·5	0·097
120	+17	1·87	0·67	71·5	0·106

* The pituitrin used was that manufactured by Parke Davis and Co.

Exp. 2 (*a*). Respiratory metabolism in diabetic patient on low diet, after intravenous injection of 20 g. of dextrose.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	− 9	1·27	0·72	36·66
1–7	Dextrose intravenously			
10	− 6	1·30	0·84	42·0
20	− 4	1·27	1·00	52·5
30	− 7	1·14	0·82	42·5
40	−24	1·09	0·72	37·0
50	−20	1·11	0·72	42·0

Exp. 2 (*b*). Same as preceding + 1 cc. pituitrin administered subcutaneously at end of dextrose injection.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	4	1·49	0·67	38·5
1–7	Dextrose intravenously + pituitrin subcutaneously			
10	+ 6	1·53	0·64	38·0
20	7	1·56	0·65	38·5
30	+ 7	1·6	0·65	39·0
40	−16	1·18	0·75	37·0
50	−11	1·25	0·74	38·0

Exp. 3 (*a*). Respiratory metabolism in normal male after intravenous injection of 20 g. of dihydroxyacetone.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	− 7	1·59	0·83	29·35
1–7	Dihydroxyacetone intravenously			
1–3	+15	1·94	0·87	42·0
3–6	+50	2·51	0·92	61·5
10	+49	2·54	0·86	57·0
20	+36	2·35	0·81	51·0
30	+12	1·91	0·84	47·0
40	+ 7	1·85	0·80	46·5
60	+ 3	1·80	0·76	40·25

Exp. 3 (*b*). Same as preceding + 1 cc. pituitrin.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	+ 5	1·84	0·77	40·2
1–7	Dihydroxyacetone intravenously + pituitrin subcutaneously			
1–3	+10	1·91	0·78	40·5
3–6	+32	2·25	0·86	50·4
10	+17	1·97	0·89	52·25
20	−15	1·99	0·81	46·5
30	+35	2·40	0·71	48·0
40	+16	1·98	0·84	54·5
70	+19	2·01	0·89	52·0
100	+ 8	1·88	0·76	38·75

Exp. 4 (*a*). Respiratory metabolism, blood-sugar and inorganic blood-phosphate in normal male after injection of 15 units of insulin subcutaneously.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	−10	1·34	0·86	38·5	0·100	3·428
Insulin 15 units subcutaneously						
15	−18	1·20	0·92	37·0	0·095	3·387
30	− 2	1·45	0·87	43·0	0·085	3·170
45	−11	1·40	0·85	39·25	0·079	2·928
60	− 9	1·35	0·88	38·5	0·079	2·991

Exp. 4 (*b*). Respiratory metabolism in normal male after subcutaneous injection of 1 cc. of pituitrin.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	−20	1·20	0·72	36	0·099	3·377
Pituitrin subcutaneously						
10	−12	1·29	0·94	44	0·107	3·172
20	−10	1·34	0·85	42	0·112	2·906
40	−13	1·32	0·78	39·5	0·113	2·777
50	−15	1·31	0·79	37	0·107	2·777

Exp. 5 (*a*). Respiratory metabolism in normal male after subcutaneous injection of 10 units of insulin.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	− 3	1·24	0·95	37·2
Insulin subcutaneously				
10	+31	1·76	0·73	38·2
20	+20	1·62	0·72	38·2
30	+19	1·58	0·80	36·0
40	+17	1·58	0·72	34·7
50	+ 7	1·36	0·78	30·5
80	+124	2·41	0·71	56·9*

* Symptoms of hypoglycaemia.

Exp. 5 (*b*). Same subject as preceding (5 *a*). Respiratory metabolism after subcutaneous injection of 1 cc. of pituitrin.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	−10	1·21	0·71	25·1
Pituitrin subcutaneously (injection painful)				
10	− 6	1·27	0·72	26·1
20	−19	1·06	0·81	25·0
30	−19	1·09	0·74	25·1
40	−12	1·17	0·77	25·2
70	−13	1·16	0·76	26·7

Exp. 5 (*c*). Same subject as in two preceding experiments (5 *a* and 5 *b*). Respiratory metabolism after simultaneous injection of insulin (10 units) and pituitrin (1 cc.) subcutaneously.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	− 2	1·33	0·72	38·5
Insulin and pituitrin subcutaneously				
10	− 5	1·28	0·74	38·5
20	− 2	1·35	0·70	39·5
30	+17	1·58	0·71	40·0
40	+ 1	1·31	0·89	42·5
70	− 9	1·21	0·77	37·8

Exp. 6 (*a*). Respiratory metabolism in normal male after subcutaneous injection of 15 units of insulin.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	−3	1·41	0·73	34·75	0·104	2·906
Insulin 15 units subcutaneously						
10	−6	1·36	0·72	31·72	0·104	2·777
20	+5	1·51	0·74	36·5	0·074	2·765
30	−2	1·41	0·77	37·6	0·066	2·687
40	−1	1·42	0·73	38·5	—	2·705

Exp. 6 (*b*). Same subject as preceding (6 *a*). Respiratory metabolism in normal male after 1 cc. pituitrin subcutaneously.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	−3	1·32	0·81	34·5
Pituitrin subcutaneously				
15	−5	1·27	0·77	35·0
25	+5	1·386	0·86	36·0
35	+1	1·380	0·73	34·5

Exp. 6 (*c*). Same subject as preceding experiments (6 *a* and 6 *b*). Respiratory metabolism, blood-sugar and inorganic blood-phosphate in normal male after subcutaneous injection of insulin, 15 units, followed 7 mins. later by pituitrin 1 cc.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	+ 6	1·61	0·74	40·75	0·111	2·928
Insulin subcutaneously						
6	+11	1·7	0·79	42·25	0·111	2·875
7	Pituitrin subcutaneously					
22	+ 4	1·5	0·96	46·25	0·100	2·823
30	+11	1·66	0·85	45·5	0·104	2·703
50	−14	1·25	1·1	43·5	0·111	—
65	−18	1·42	1·0	40·75	0·111	2·652

Exp. 6 (*d*). Same as preceding but pituitrin given 25 mins. after insulin. Respiratory metabolism.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	−16	1·19	0·81	29·5
Insulin subcutaneously				
10	−14	1·26	0·78	28·5
25	Pituitrin subcutaneously			
35	−13	1·21	0·86	33
45	5	1·32	0·86	39·5
55	9	1·27	0·85	34
65	−11	1·25	0·84	32

Exp. 6 (*e*). Respiratory metabolism, blood-sugar and inorganic blood-phosphate in normal male (different subject from preceding) after insulin, 15 units, followed 62 mins. later by pituitrin 1 cc.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	+11	1·76	0·76	31	0·100	3·515
Insulin subcutaneously						
30	+30	2·06	0·78	37·5	0·082	2·928
60	+24	1·17	0·76	36	0·076	2·510
62	Pituitrin subcutaneously					
64	+30	2·01	0·86	42·35	0·076	2·130
74	+32	2·07	0·80	43·25	0·079	2·162
84	+18	1·85	0·81	36·2	0·082	2·381
94	+26	2·01	0·75	37·52	0·086	2·343
104	+11	1·75	0·77	33·75	0·086	2·421

Exp. 7 (*a*). Respiratory metabolism, blood-sugar and inorganic blood-phosphate in normal subject after subcutaneous injection of 0·75 cc. of adrenaline (1 in 1000).

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	+ 8	1·99	0·81	40·5	0·091	3·652
Adrenaline subcutaneously						
10	+35	2·41	0·94	57·0	0·117	3·255
20	+40	2·59	0·80	54·25	0·133	3·181
30	+45	2·69	0·78	53·0	0·133	3·232
40	− 7	1·69	0·93	41·25	0·25	3·308

Exp. 7 (*b*). Respiratory metabolism in normal male after 0·75 cc. of adrenaline (1 in 1000) subcutaneously.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	−13	1·39	0·76	42
Adrenaline subcutaneously				
10	+ 8	1·62	0·84	53·7
20	+ 8	1·6	0·82	50·25
30	− 5	1·5	0·76	47
40	− 8	1·34	0·86	49
50	− 9	1·39	0·75	43

Exp. 7 (*c*). Same as 7 (*b*). Dextrose 20 g. injected intravenously and adrenaline 0·75 cc. subcutaneously.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	−27	1·3	0·72	38
Dextrose intravenously; adrenaline subcutaneously				
10	+42	2·15	0·82	72
20	+ 7	1·63	0·78	54
30	− 4	1·46	0·80	48
40	−18	1·25	0·76	38
50	−16	1·28	0·78	40

Exp. 8. Respiratory metabolism, blood-sugar and inorganic blood-phosphate in a patient suffering from exophthalmic goitre, after oral administration of 50 g. of dextrose.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	+ 52	1·58	0·85	46·75	0·075	3·947
Dextrose orally						
30	+102	2·17	0·81	51·25	0·145	3·448
60	+ 48	1·88	0·71	58·0	0·205	3·355
90	+ 52	1·86	0·85	54·5	0·162	3·488
120	+ 37	1·71	0·78	57·5	—	—

B. Animal experiments.

Exp. 1. Rabbit 2340 g. Effect of pituitrin alone on blood-sugar and inorganic blood-phosphate.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·124	3·472
Pituitrin 0·5 cc. intravenously		
15	0·159	3·874
35	0·124	3·204

Exp. 2 (*a*). Rabbit 2950 g. Effect of dihydroxyacetone alone.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·145	4·166
Dihydroxyacetone 2·5 g. intravenously		
15	0·288	3·424
30	0·247	3·100
45	0·236	2·976

Exp. 2 (*b*). Same animal as Exp. 2 (*a*). Effect of pituitrin and dihydroxyacetone together.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·111	2·702
Pituitrin 0·5 cc. + dihydroxyacetone 2·5 g. intravenously.		
15	0·255	3·120
30	0·238	3·030
45	0·217	2·630

Exp. 3 (*a*). Rabbit 2500 g. Effect of dextrose alone.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·97	3·900
Dextrose 2·5 g. intravenously		
15	0·186	3·676
30	0·115	3·472
45	0·084	3·378

Exp. 3 (*b*). Same animal. Effect of dextrose and pituitrin together.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·132	3·570
Pituitrin 0·5 cc. + dextrose 2·5 g. intravenously		
15	0·302	3·56
30	0·164	3·444

Exp. 4 (*a*). Rabbit 1390 g. Effect of dihydroxyacetone and pituitrin together, using smaller dose of pituitrin than in preceding experiments

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·113	3·387
Pituitrin 0·1 cc. + dihydroxyacetone 1·39 g. intravenously		
20	0·294	2·876
45	0·168	2·763

Exp. 4 (*b*). Same animal as Exp. 3 (*a*). Effect of dextrose and pituitrin together in similar doses.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·120	3·402
Pituitrin 0·1 cc. + dextrose 1·39 g. intravenously		
20	0·175	3·735
45	0·121	3·206

Exp. 5 (*a*). Rabbit 3000 g. Effect of dihydroxyacetone and pituitrin together.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·125	3·786
Pituitrin 0·1 cc. + dihydroxyacetone 2·5 g. intravenously		
15	0·264	3·204
30	0·187	3·110

Exp. 5 (*b*). Same animal. Effect of dextrose and pituitrin together in similar doses.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·115	3·2110
Pituitrin 0·1 cc. + dextrose 2·5 g. intravenously		
15	0·285	3·7668
30	0·135	3·1865

Discussion.

The above experiments appear to show that pituitrin has a marked inhibitory action upon the metabolism of carbohydrates. Thus Exp. 1 (*a*) shows the typical changes which occur in the respiratory metabolism and blood-sugar in the healthy individual following the administration of 20 g. of dextrose intravenously. There is first a gradual rise in the heat production and oxygen consumption, the R.Q. and the respiratory volume, maximal values being attained in approximately 20–30 minutes. (The high initial R.Q. in this experiment may have been due to overbreathing.) The blood-sugar following the injection falls rapidly, reaching the base line in 30–40 minutes. When the same amount of dextrose was administered to this individual along with pituitrin, injected subcutaneously, there was little if any rise in the heat production, oxygen consumption, R.Q. or respiratory volume by the time these would have been at their height in the control. In fact, save for an initial increase in the metabolic rate and oxygen consumption, for which changes in blood flow resulting from the injection of fluid may have been responsible, there was remarkably little alteration in the metabolism for the first 30 or 40 minutes. After this, however, coinciding with the passing off of the vaso-constriction, as evidenced by diminution in the pallor of the skin, there was a rise in all these values with the exception of the R.Q. which fell a little when the heat production and oxygen consumption were at a maximum. The R.Q. rose to its highest at a later period. It is interesting to note that the blood-sugar fell very slowly during the period when the respiratory metabolism showed little change, being still well above the base line at the end of 40 minutes. After this, however, it fell while the metabolism rose.

In Exp. 2, which was performed upon a diabetic patient on a low diet, the changes are less striking but are of a similar character. The R.Q., which was remarkably low, here attained its maximum after all the other values had fallen.

The effect of pituitrin upon the changes in respiratory metabolism which follow the intravenous administration of dihydroxyacetone is equally striking. In spite of the administration of a dose of pituitrin sufficient almost completely to annul the rise in metabolism due to dextrose during the first 20 minutes of the experiment, there was still a considerable rise in the metabolism during this period; in fact the actual rise was even greater than that obtained with dextrose alone. Nevertheless, the rise in metabolism was not nearly as great as with dihydroxyacetone alone, the percentage inhibition due to pituitrin being just as great as in the case of dextrose. In addition to the inhibition of the metabolism, however, the form of the curves for heat production and oxygen consumption were entirely altered, resembling in certain respects those obtained with dextrose alone. Thus, whereas dihydroxyacetone alone caused an immediate rise in metabolism with maximal values

soon after the completion of the injection, followed by a rapid fall to almost normal at the end of 30–40 minutes, pituitrin given along with dihydroxyacetone inhibited the initial acceleration in metabolism (see 10 minutes' sample), there being instead a gradual rise reaching a maximum in 30 minutes. The R.Q., however, varied inversely with the oxygen consumption and heat production during this period. A slight secondary rise in the metabolic rate, oxygen consumption, R.Q. and respiratory volume was observed at the end of 70 minutes.

In the experiments in which insulin and pituitrin were injected together, the results were not nearly so clear cut as when pituitrin was given along with carbohydrate, owing to the changes in respiratory metabolism produced by either hormone during the first 30–40 minutes after their administration being so slight as to be of doubtful significance. McKinlay [1921], Bowman and Grabfield [1926] and Castex and Schteingart [1926] report a rise in metabolism of about 10 % occurring 20–30 minutes after the administration of pituitrin alone; but our results are not so uniform. Thus, in Exp. 4 (*b*) there was a slight rise in the heat production, oxygen consumption and R.Q.; in Exp. 6 (*b*) there was a preliminary fall during the first 15 minutes, followed by a rise, while in Exp. 5 (*b*) there was a more prolonged fall in the heat production and oxygen consumption, but not in the R.Q. The slight rise in metabolism obtained in the 10 minutes' sample in this experiment may have been occasioned by the pain of the injection.

These irregular results may be due to pituitrin on the one hand stimulating intestinal peristalsis, which would tend to increase the oxygen consumption, and on the other hand depressing the metabolism in other tissues. The effect upon the respiratory metabolism would depend upon the time relationship and relative potency of these two factors. In both Exps. 5 (*c*) and 6, (*c*) in which insulin and pituitrin were injected either simultaneously or within a comparatively short interval of one another, pituitrin seemed to inhibit the rise in metabolism or cause a fall at the time of the corresponding observations in the control with insulin alone. Since, however, pituitrin by itself caused a fall in metabolism at these times in this particular series of experiments, the effect of the administration of the two hormones together was apparently such as might have resulted from a combination of their independent actions.

When pituitrin was injected at a longer interval (half an hour to an hour) after insulin (Exps. 6 (*d*) and 6 (*e*)) a slight rise in the heat production and R.Q. was observed. This may possibly be connected with the liberation of glucose from the liver or with the contractions of the stomach and intestine which are more readily excited in hypoglycaemia. (Hunger pains are common during hypoglycaemia.)

Our findings regarding the effect of pituitrin upon the blood-sugar are in agreement with those of other observers, namely, a delay in the disappearance of injected dextrose from the blood [Hines, Leese and Boyd, 1927; Achard, Ribot and Binet, 1919], inhibition of hypoglycaemia or a return of the blood-

sugar towards the normal level in hypoglycaemic animals after insulin [cf. Burn, 1923; Olmstead and Logan, 1923; Moehlig and Ainslee, 1925; Lawrence and Hewlett, 1925; Geiling and Britton, 1927], while injection of pituitrin alone caused a transient but inconstant rise in the blood-sugar during the first 30 minutes after its administration [cf. Borchardt, 1908; Goetsch, Cushing and Jackson, 1911; Parton and Katz-Klein, 1921; Lindlau, 1928; Blotner and Fitz, 1928].

With regard to phosphate metabolism, pituitrin alone in the doses employed in the human subject caused a slight fall in the percentage of inorganic phosphate in the blood, but given in relatively large doses to rabbits, it caused a marked but transient rise. When administered to either human beings or rabbits with hypoglycaemia and a low inorganic blood-phosphate resulting from insulin administration, it caused the percentage of inorganic blood-phosphate to rise. Similar results were obtained with adrenaline as with pituitrin.

The results so far reviewed, taken as a whole, might at first sight seem to bear out the contention that pituitrin inactivates insulin, thereby bringing about, temporarily, a state of affairs analogous to that obtaining in diabetes. Hyperglycaemia, delayed disappearance from the blood of injected carbohydrate, diminished response in respiratory metabolism following administration of carbohydrate and an increased percentage in inorganic blood-phosphate, all of which may follow the injection of suitable doses of pituitrin, are also features characteristic of the diabetic condition. Taken in conjunction with the observation that pituitrin seems to inhibit any slight rise in metabolism following administration of insulin, the evidence in support of a direct antagonism would at first sight appear to be strong and to have the advantage of economy of hypothesis. Nevertheless, in certain respects this explanation does not appear to be wholly satisfactory. Thus, pituitrin, except in the large doses employed in rabbits, did not, when given alone, cause any rise in the inorganic blood-phosphate, but rather a slight fall; moreover, in spite of being given in doses sufficient completely to prevent the fall in blood-sugar following insulin administration in the human subject, it did not prevent the inorganic blood-phosphate from falling (Exp. 6 (*c*)), and, although the fall may have been temporarily checked, the blood-sugar remained at the normal level while the level of inorganic blood-phosphate continued to decline.

Again, pituitrin caused no definite fall in the respiratory quotient, but more often a rise.

Further, in the experiment with dihydroxyacetone and pituitrin (Exp. 3 (*b*)), the curves of metabolism fail to reproduce the features of those obtained in diabetes. In the latter [cf. Lambie and Redhead, 1927] the heat production, oxygen consumption and R.Q. attained their maxima together soon after the injection, whereas in the experiment referred to the heat production and oxygen consumption gradually rose, reaching their height at the end of 30 minutes, while the respiratory quotient was at its highest in 10 minutes and declined *pari passu* with the rise in the metabolic rate.

In view of these differences between the effects of deficiency of insulin and the alleged inactivation of insulin by pituitrin, it is necessary to consider other possible explanations of the phenomena. Krogh [1922] has shown that pituitrin not only constricts the arterioles, but markedly increases the contractile tonus of capillaries, the effect of which would be to diminish the capillary bed. This would lead to a decreased rate of diffusion of injected material out of the blood into the tissues in which it was to be metabolised and in this way it may be possible to explain the delay in the rise in metabolism with dihydroxyacetone or dextrose, and the delay in the disappearance of sugar from the blood after intravenous injection. The occasional sudden rise in metabolism during and immediately after intravenous injection may have been the result of a temporary increase of blood flow due to the volume of fluid injected, especially since the fluid was hypertonic. The experiments of Hines, Leese and Boyd [1927] did not show any definite change in the heat production or R.Q. as a result of injecting pituitrin during continuous transfusion of dextrose, but this may have been due partly to the very large quantity of dextrose injected and to the fact that there was a continuous transfusion of fluid into the circulation. Their results are therefore not inconsistent with our own, but are due to the different conditions of the experiments.

The depression in metabolism sometimes observed following injection of pituitrin alone may again be due to diminished blood flow through the muscles, while the subsequent rise may perhaps be related to the contraction of non-striped muscle, particularly that of the intestine (borborygmi being frequently audible) and to increased work of the heart.

The rise in inorganic blood-phosphate noted after injection of large doses of pituitrin might also be explainable as a result of local asphyxia in the muscle cells, while increase in the blood-sugar may perhaps be related to changes in the portal circulation [cf. Clark, 1928].

It appears reasonable to expect that drugs, which, like pituitrin and adrenaline, alter the blood flow through organs, particularly those which, like muscle, are important seats of heat production, should be capable of modifying metabolism, and, if at the same time they cause change in the tonus of muscle or the work of the heart, further complications are to be anticipated. The metabolic changes which result from the administration of adrenaline should therefore be considered in this light also, but further experiment is necessary before definite conclusions can be drawn. Krogh [1922] points out that adrenaline differs from pituitrin in having a more powerful action on the arterioles than upon the capillaries, but all arterioles in the body are not equally affected. Hoskins, Gunning and Berry [1916] and Gunning [1917] have brought forward evidence to show that adrenaline in small doses causes blood to be diverted from the splanchnic area and skin while the blood flow through the voluntary muscles and myocardium is increased. This, together with the increased work of the heart, may be responsible for the

marked and sudden rise in metabolism following the administration of adrenaline alone, but it remains an open question as to how the metabolic processes in and the blood flow through the muscles are causally related.

Our experiments do not support the view that the rise in metabolism is to be ascribed to the hyperglycaemia. In the first place, the rise in metabolism following injection of adrenaline is very great even with a slight hyperglycaemia, whereas with a much greater hyperglycaemia produced by intravenous injection of dextrose there was less increase in the metabolism [cf. Boothby and Sandiford, 1921]. The time relationship between hyperglycaemia and the change in respiratory metabolism is also quite different in the two cases. Moreover, the rise in metabolism which occurs after injecting adrenaline is much more rapid than after intravenous injection of dextrose. Thus, 10 minutes after administration of adrenaline in Exps. 7 (*a*) and 7 (*b*) there was a 20 % rise in the metabolism at a time when the blood-sugar had risen (cf. Exp. 7 (*a*)) only slightly above the fasting level, whereas 10 minutes after injection of 20 g. of dextrose intravenously (Exp. 1 (*a*)) there was practically no increase in respiratory metabolism although there was a definite hyperglycaemia. It may be suggested as a possible explanation of these results that the dextrose liberated by the liver is more immediately available for combustion than ordinary dextrose, but apart from the lack of evidence to support this view, there is the observation that in Exp. 7 (*a*) with adrenaline the blood-sugar continued to rise even when the metabolic rate was falling. There appears, therefore, to be no high degree of correlation between hyperglycaemia and heightened metabolism after administration of adrenaline.

As to the method by which adrenaline causes hyperglycaemia, stimulation of the endings of the splanchnic nerves in the liver, changes in the portal circulation [cf. Clark, 1928], and perhaps the breaking down of muscle-glycogen, may all play a part; but there is no need to assume a direct antagonism to insulin.

The influence of adrenaline upon phosphorus metabolism has been investigated by Allan, Dickson and Markowitz [1924] and the results obtained were similar to those produced by insulin, indicating that, as in the case of pituitrin, there was no antagonism between the two in this respect.

The effect of simultaneous injection of adrenaline and insulin upon the respiratory metabolism is such as would be expected to result from the combined effect of each separately. Thus, in Exp. 7 (*b*), the percentage rise in calorie consumption 20 and 30 minutes after adrenaline alone was 21 % and 8 % respectively. In most experiments with 20 g. of dextrose alone, intravenously, the percentage rise at these periods is 5 % and 10 % respectively. The combined effects of dextrose and adrenaline would therefore give + 26 % and + 18 %. In Exp. 7 (*c*) with both adrenaline and dextrose together the actual figures obtained were + 34 % and + 23 %; there is, therefore, no indication of antagonism here.

As in the case of adrenaline, no evidence could be obtained of any

inhibitory effect of excessive thyroid activity upon the metabolism of dextrose, but rather the reverse. Thus, in Exp. 8 the metabolic rate rose to over 100 % 30 minutes after the administration of 50 g. of dextrose by the mouth to a patient with exophthalmic goitre.

The facts so far brought forward, therefore, afford no definite proof of there being a direct inactivation of one hormone by the other, and, until the changes in the circulation can be excluded as the cause of the apparent antagonism, it appears unnecessary to assume that any more direct and specific action is involved. It is true that in certain forms of pituitary disease (acromegaly) there is often diminished sugar tolerance, or even a condition indistinguishable from true diabetes, together with marked resistance to insulin [Ulrich, 1928], but no obvious disturbance of the circulation is present in such cases. The assumption that the low carbohydrate tolerance is due to excessive activity of the posterior lobe of the pituitary body is, however, unwarranted, since the glycosuria occurs in connection with a definite type of tumour of the anterior lobe, namely, the eosinophil adenoma, and not with other tumours, although the latter are more numerous [cf. Davidoff and Cushing, 1927]. Although nothing is at present known regarding the effect of repeated small doses of pituitrin administered over a long period, the fact that the apparent inhibition of carbohydrate metabolism has so far only been obtained with doses sufficiently large to cause obvious circulatory disturbances (*e.g.* blanching in man), makes the resemblance to the clinical pituitary glycosuria incomplete. Since it has been impossible to settle the question of the nature of the antagonism between pituitrin and insulin the significance to be attached to the peculiar behaviour of dihydroxyacetone in the above experiment must likewise remain an open question. The fact that it caused a greater rise in metabolism than dextrose, and brought about a fall in inorganic blood-phosphate in rabbits when dextrose failed to do so after the administration of pituitrin, is merely in conformity with previous findings [Lambie and Redhead, 1927].

Summary.

1. In the human subject pituitrin frequently causes a transient fall in metabolism followed by a rise. Sometimes only a rise is observed. In small doses it causes a slight fall in the inorganic phosphate of the blood.

2. Pituitrin apparently inhibits the slight rise in metabolism which occurs after the administration of insulin; but the changes produced by either insulin or pituitrin are not sufficiently marked to be of much significance.

3. Pituitrin delays and reduces the rise in metabolism and the fall in blood-sugar following the intravenous administration of dextrose or dihydroxyacetone.

4. Pituitrin in small doses sufficient to prevent the fall in blood-sugar resulting from administration of 15 units of insulin did not prevent the fall in inorganic blood-phosphate.

5. Pituitrin administered in large doses to rabbits causes a transitory rise in the inorganic blood-phosphate and for a short period inhibits the fall in inorganic phosphate resulting from administration of dextrose or dihydroxyacetone. A dose of pituitrin which just suffices to prevent the fall in inorganic phosphate following administration of dextrose may fail to overcome the fall due to dihydroxyacetone.

6. In animals rendered hypoglycaemic as a result of insulin administration pituitrin causes the blood-sugar and inorganic blood-phosphate to return to the normal level or even to exceed it.

7. Adrenaline administered alone causes a marked and rapid rise in metabolism which is not solely dependent upon the rise in blood-sugar.

8. Adrenaline does not inhibit the rise in metabolism following intravenous administration of dextrose.

9. Adrenaline administered in large doses to hypoglycaemic animals causes a rise in inorganic blood-phosphate.

10. The question is discussed as to how far the effects of pituitrin and adrenaline upon metabolism result from the changes in the circulation and distribution of blood in the body or whether some more direct and specific antagonism to insulin is to be assumed. It is concluded that experiments so far recorded have not proved unequivocally that such a direct action exists.

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LXXI. THE REACTION OF AZINE COMPOUNDS WITH PROTEOLYTIC ENZYMES.

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THE formation of precipitates when various azine dyes are added to solutions of proteolytic enzymes has been remarked by a number of observers. In some cases, moreover, it has been shown that the mother liquors have lost their protease activity and that this has, in part, been transferred to the precipitates [Holzberg, 1913; Wood, 1918; Marston, 1923; Forbes, 1927]. The observations and conclusions of Marston are of particular interest. Marston found that safranine was a specific precipitant for all the protease activities he tested—pepsin, trypsin, erepsin, yeast protease, papain. The dye did not, however, remove other non-proteolytic enzymes from solution. Since, moreover, other azonium salts, azines, and eurhodines behaved similarly towards trypsin, Marston concluded that he was observing a specific interaction between the azine nucleus and the structure peculiar to a protease. He further fortified this argument by drawing an analogy between the azine ring as represented by the various dyes employed and the piperazine ring which he believes to be a dominant feature of the protein molecule. Robertson [1928], indeed, has employed Marston's argument to support the diketopiperazine structure of protein. Now the view that the azine nucleus found in these dyes resembles the heterocyclic ring of a diketopiperazine is open to serious criticism upon structural grounds and makes the acceptance of the full implications of Marston's argument difficult. On the other hand, there is no doubt that valuable opportunities are opened by the demonstration that a simple well-defined structure reacts with a group of enzymes in a specific way which parallels the catalytic specificity of that group of enzymes.

In particular, Marston's observations suggested to us an opportunity for the examination of the kinetics of protease activity by means of a quantitative study of the anticatalytic effect of azine compounds on the activity of the protease. At the same time it was hoped to assemble data which would permit the precise use of this reaction as an economical means of purifying protease preparations.

The poisoning of protease activity by azine dyes.

Protease activity was determined by the viscosity method of Northrop and Hussey [1923], where the measure of activity is the time taken to produce a definite degree of viscosity change in a solution of gelatin. Results were conveniently reproducible and a relative measure of activity accurate to within 10 % was obtained when all the necessary precautions were observed. The comparison of activities was most reliable if the different enzyme preparations were separately diluted to produce approximately similar rates of viscosity change. A dilution which produced half of the total viscosity change in 10 to 30 minutes was adopted as being most convenient. Comparison of activities determined on substrate samples of markedly different initial viscosity is not strictly valid, but is useful qualitatively.

Viscosity determinations at 30° were made in viscosimeters standardised against distilled water and, after each determination, cleaned with chromic acid overnight. The substrate solutions were 2·75 % solutions of isoelectric gelatin [Loeb, 1922] adjusted with phosphoric acid or phosphate to a p_H of 2·7 for pepsin and 7·5 for trypsin determinations. After addition of a trace of thymol, these bulk solutions were stored in small bottles in the refrigerator. For use each bottle was warmed to melt the gelatin and maintained at 30° for at least 2 hours. Then, as required, samples were removed, mixed at zero time with one-twentieth volume of the enzyme preparation, and pipetted in 7 cc. amounts to the viscosimeters, every precaution being taken to minimise cooling of the solutions below 30°. Initially, determinations were made in duplicate until the precaution was found unnecessary.

Table I. *Poisoning of protease activity by azine dyes.*

Enzyme	Azine dye	[E]	[D]	t mins.	Gelatin sample
Dog's gastric juice	Safranine	0·002	—	26	a
		0·002	0·01	27	a
		0·002	0·05	37	a
		0·002	0·2	69	a
		0·002	0·6	100	b
Benger's trypsin	Neutral red	0·0015	—	29	c
		0·0015	0·00025	26	c
		0·0015	0·0012	26	c
		0·0015	0·0063	25	c
	Safranine	0·0015	0·0063	25	c
		0·0015	—	44	d
		0·0015	0·0063	43	d
		0·0015	0·025	39	d
		0·0015	0·1	47	d
		0·0015	0·2	70	d
		0·0015	0·6	90	d

[E] = concentration of enzyme in 100 cc. of the digestion mixture in terms of total solids (g.) of the original preparation.

[D] = concentration of dye (g. %) in the digestion mixture.

t = time, corrected to a constant arbitrary enzyme concentration, for 50 % viscosity change in the gelatin.

p_H for pepsin experiments 2·7, for trypsin experiments 7·5.

In Table I and Fig. 1 are summarised the results of experiments on gastric juice from a dog's Pavlov fistula (0·42 % solids) and upon a highly active

pancreatic extract (6·1 % solids) kindly prepared for us by Benger's Food Products Ltd.

It is obvious that dye concentrations of even 0·05 to 0·1 % in no way retard the hydrolysis of the gelatin by the enzyme. Moreover, it should be pointed out that the concentration of dye at which retardation does definitely occur is the critical concentration for immediate precipitation of the enzyme complex (though precipitation occurs in 24 hours with all dye concentrations above 0·006 %). Therefore, not only is there no poisoning with small concentrations of dye, but such diminution in activity as is observed with high concentrations occurs only upon visible separation of a gelatinous precipitate in the viscosimeter. The artificial effect of such a precipitate is obvious, and appears in the rather erratic viscosity curves at these concentrations (Fig. 1). Hence the indicated decrease in activity may well be merely apparent.

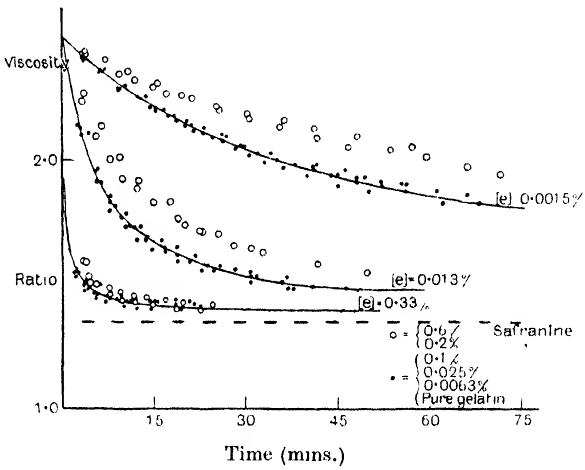

Fig. 1. Poisoning test (safranine on Benger's trypsin) $p_H = 7 \cdot 5$.

To test this point the diastase activity of the Benger's pancreas extract was investigated with starch in the presence of varying concentrations of dye. Though results were more erratic, an effect running quite parallel to the above was obtained, so that, since diastase activity is not precipitated by azine dyes [Marston, 1923], any retardation would appear to be artificial and dependent at least predominantly on the gelatinous nature of the precipitate. Complete confirmation of this view was received, at least for the trypsin experiments, when it was discovered later that the tryptic activity of the Benger's extract is not significantly precipitated by azine dyes.

These results do not deny a specific chemical reaction between the dye and the catalytically active grouping of the protease. They do suggest, however, that the hypothetical complex formed is so largely dissociated in solution that the active mass of free enzyme is unaffected by such concentrations of dye as will cause an ultimate separation of the insoluble complex.

Further information was sought by direct studies of the protease activities of azine precipitates and of the mother liquors from which these precipitates have been separated.

The precipitation of pepsin and trypsin by neutral red.

The factor which has limited former studies of these precipitates has been the difficulty of redissolving the active material. Only in the case of pepsin had this been found possible [Marston, 1923; Forbes, 1927], advantage being taken of the fact that in dilute solutions of strong acids the precipitate redissolves and the dye may be extracted by such solvents as amyl alcohol. Even in this case, however, emulsification during extraction rendered quantitative recovery of the enzyme difficult.

Now Marston found that the leuco azine bases do not form precipitates with enzymes. We find further that a reducing agent such as sodium hyposulphite will redissolve an existing precipitate. Moreover, the reducing agent does not destroy the activity of the protease present. A simple method of regaining the precipitated enzyme in solution at once suggests itself. The precipitate is removed from the reaction mixture by centrifuging, washed with 0·1 % safranine and suspended in an appropriate buffer. Sodium hyposulphite is added and the leuco-base extracted with benzene in a vessel protected from oxygen. A useful extraction device which avoids any emulsification consists of a shallow cylinder in which a thin layer of redissolved precipitate covered with a layer of benzene is gently and slowly rocked. Two changes of solvent give efficient extraction, the whole process being complete in an hour.

A still simpler method of extracting the precipitate—in the case of trypsin—is based upon the observation that a neutral red precipitate will redissolve in solutions of p_H more alkaline than about 7, *i.e.* when the basic dissociation of neutral red is suppressed. It is necessary therefore only to precipitate the preparation at p_H 6, adjust the solution to p_H 9, and extract the yellow dye with benzene. The same rocking device may be employed.

The various reagents and manipulations involved in these methods have no destructive effect upon either pepsin or trypsin. This was established by comparing the activity of an enzyme solution before a precipitate was formed in it by addition of dye and after the precipitate had been redissolved in its own mother liquors and extracted by one of the methods described.

In estimating the activity remaining in the mother liquors it proved to be unnecessary to carry out a preliminary extraction of the dye since the further degree of dilution involved in carrying out the activity test reduced the concentration of dye far below that which had been found to give significant "poisoning" (Table I).

Protease activity of the enzyme precipitates and supernatant liquors.

In these investigations the enzyme solutions were buffered at p_H 3·9 (acetate buffer) for pepsin and at p_H 6·0 (phosphate buffer) for trypsin. p_H determinations were made with a Hildebrand hydrogen electrode slightly modified to eliminate frothing. Samples of the buffered preparations were then mixed with varying concentrations of dye, allowed to flocculate for 15 hours in the refrigerator, centrifuged for 20 minutes in a high speed centrifuge, and the supernatant liquors decanted from the precipitate. The precipitate was dried to constant weight in a steam-oven, and the extent of precipitation of the protease activity was estimated by the loss of activity in the supernatant liquors. In this way the loss of activity was compared with the amount of precipitate carrying it down and with the concentration of the precipitating dye. The data appear in Table II.

Table II. *Protease activity of the supernatant liquors.*

Enzyme	Dye	$[D]$	$\frac{100w}{S}$	t (supernatant) mins.	Gelatin sample
Harrington's pepsin	Neutral red	—	Not determined	11	e
2 % solution		0·024	,,	50	e
		0·045	,,	80	e
		0·083	,,	150	e
Dog's gastric juice	Safranine	—	—	6	f
$[E]=0·195$		0·005	—	6·5	f
		0·01	9	8	f
		0·02	10	16	f
		0·05	19·5	144	f
		0·1	23·5	550	f
		0·2	24	700	f
		0·5	Not determined	800	f
Harrington's trypsin	Safranine	—	,,	7	g
3 % solution		1·0	,,	10	g
Benger's trypsin	Safranine	—	—	29	h
$[E]=2·6$		0·1	5	28	h
		0·5	12	29	h
		1·0	17	28	h

$[E]$ = concentration of enzyme in 100 cc. of the precipitation mixture in terms of total solids (g.) of the original preparation.

$[D]$ = concentration of dye (g. %) in the precipitation mixture.

$\frac{100w}{S}$ = dry weight of precipitate expressed as percentage of the total solids of the original preparation.

$[E]$ and $[D]$ are diluted 20 times (or 2000 times for Benger's trypsin) in the activity tests.

A saturated aqueous solution of safranine contains just above 1 % of dye.

The data for pepsin precipitation are in general agreement with Marston's views, since loss of activity runs parallel with both the concentration of dye and the weight of precipitate. However, it is noticed that, though the critical dye concentration for removal of peptic activity from solution is 0·02 to 0·1 % with practically complete removal at 0·2 %, yet by weight 38 % of the precipitate at 0·2 % has already been precipitated in a dye concentration of

only 0·01 %. This anomaly becomes more significant when considered in the light of the trypsin results, for here we find the quite unexpected fact that, though the weight of the precipitate varies definitely with the dye concentration, the activity of the supernatant liquor is not measurably reduced by loss of this precipitate. The activity of such precipitates was quite definite, but, as Table III indicates, it varied with the origin of the preparation, and was quite a negligible fraction of the total activity.

Now Marston claims to have found 64 % of the total activity of a trypsin solution in his precipitate. This variability in extent of trypsin precipitation allows of no simple explanation other than the obvious one that the precipitation does not specifically involve the enzyme. We incline to the view that trypsin preparations may or may not contain varying amounts of a substance (probably protein) with which the enzyme tends to be closely and specifically associated, and that this substance is precipitated by azine dyes removing the enzyme with it. It is of interest, therefore, that Wood [1918] reports an active preparation of trypsin which failed to give any precipitate with safranine.

Attempts were now made to encourage removal of the dye by inducing formation of some foreign precipitate in the solution. Robertson mentions the precipitation of phosphates and nucleic acid by azine dyes, so experiments were conducted in which Benger's trypsin was mixed with varying concentrations of nucleic acid and then precipitated in the usual fashion. The results were entirely negative and appear in Table III. Precipitations were performed in 0·1 % neutral red at p_H 6. Activities were determined in the supernatant liquors after extraction of the dye with benzene at p_H 9.

Table III.

(*a*) *Protease activity of the precipitates.*

(*b*) *Effect of induced precipitation.*

$[D] = 0·1$ %.

Enzyme	Nucleic acid conc. %	t mins.
10 % Harrington's trypsin	—	1·5
,, ,, extracted precipitate	—	36
Benger's trypsin, 3 % total solids	—	15
,, etc., extracted precipitate	—	6×10^4
,, ,, extracted supernatant	—	12
,, ,, ,,	0·016	13
,, ,, ,,	0·08	11·5
,, ,, ,,	0·4	12·5

The values of t given for the extracted precipitates have been corrected so as to define the activity of these extracts when diluted to the same volume as that of the enzyme solution from which the precipitates were obtained. In the case of Benger's trypsin this leads to a calculation value which has only qualitative significance indicating virtual absence of activity in the precipitate.

It is obvious that no trypsin activity has been incorporated within the bulky precipitate formed between neutral red and nucleic acid. It does not appear therefore that the presence of nucleic acid in the enzyme preparations of Marston was responsible for his results.

Effect of p_H *on precipitation of pepsin.*

Aliquots of a sample of mixed gastric juice were titrated to definite p_H values with a citric acid-citrate buffer of p_H 6, being at the same time adjusted to equal dilution. Safranine (p_H 2·6) was added, the final concentration being 0·2 % dye and 0·175 % pepsin (total solids). The solutions were left for 15 hours for flocculation, the precipitates separated in the centrifuge, and the mother liquors decanted and tested for activity. The dry weights of the precipitates were determined. The results appear in Fig. 2 and in Table IV.

Table IV. *Effect of* p_H *on precipitation of gastric juice by safranine.*

$[D] = 0{\cdot}2\ \%$. $[E] = 0{\cdot}175\ \%$.

p_H	t mins.	$\frac{100T}{t}$	$\frac{100w}{S}$	$100\left(1-\frac{T}{t}\right)$
1·0 (dye-free)	7·5	100	—	—
2·45 (dye-free)	8	95	—	—
4·0 (dye-free)	5·5	135	—	—
1·0	11	68	16	32
1·95	14·5	52	18	48
2·2	18	42	19	58
2·45	30	25	21	75
2·75	60	12·5	22	87·5
2·95	160	4·7	27	95·3
3·97	1300	0·6	65	99·4
4·85	1300	0·6	91	99·4
(From Table II)				
3·9	700 (× 1·1)	0·9	24	99·1
3·9 (dye-free)	6 (× 1·1)	109	—	—

$\frac{100T}{t}$ = the percentage of the original protease activity remaining in the supernatant liquors after precipitation at the given p_H ($T = 7{\cdot}5$).

$100\left(1-\frac{T}{t}\right)$ = the percentage of the original protease activity removed with the precipitate by precipitation at the given p_H.

It will be observed that the degree of removal of activity and the weight of precipitate both increase as the p_H rises above 1. At the same time these two factors are not closely parallel, since 88 % of the enzyme has been removed at p_H 2·75 although the weight of precipitate is only 24 % of that found at p_H 4·85. Indeed, the actual separation of insoluble matter is spread over a range of about four p_H units while the precipitation of activity is a phenomenon localised fairly definitely about p_H 2 (Fig. 3). This is of interest in view of the fact that a number of observers have reported a critical point for pepsin—isoelectric point, point of optimum adsorption, etc.—within the region of p_H 2·5 [Michaelis and Davidsohn, 1910; Northrop, 1920; Kikawa, 1926; Forbes, 1927; Fenger and Andrew, 1927]. The curve reproduced in Fig. 3 is that of a hypothetical hydrion dissociation of pepsin of pK 1·9, and

the alignment with the observed degree of precipitation of the activity may be taken to suggest that this reaction of pepsin with safranine is conditioned by the acid-base relations of the reactants. It is of course quite probable that this pK is a characteristic of the inert material with which the enzyme is

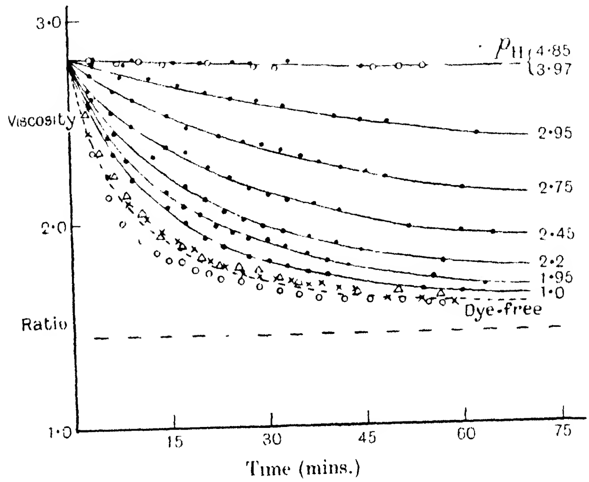

Fig. 2. Effect of p_H on precipitation of gastric juice by safranine (activity of supernatant liquors). $[D]=0{\cdot}2\ \%$. $[E]=0{\cdot}175\ \%$.

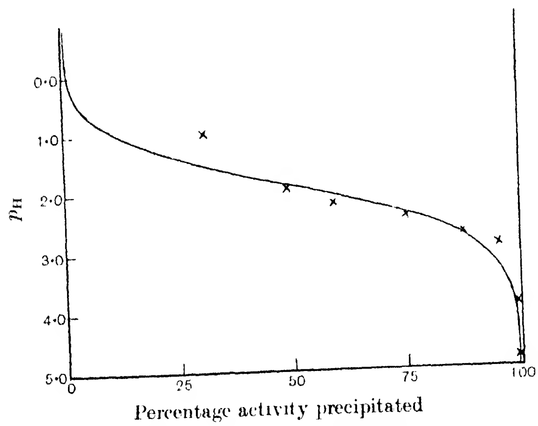

Fig. 3. Relation of precipitated activity to p_H (safranine and dog's gastric juice). The curve is that for a hypothetical hydrion dissociation of pK 1·9.

closely associated rather than of the enzyme itself. The divergence from the curve at p_H 1 may plausibly be attributed to the effect of the weak second basic group of the dye modifying the active mass of safranine at this p_H. In any case, "percentage activity" is a reciprocal term permitting considerable error below values of 50 %.

Purification of pepsin.

The following observations are of interest.

(1) An 8 % solution of a dry preparation of pepsin (Harrington and Co.) after dialysis against running water retained 95 % of its activity and lost 62 % of its solids. The neutral red precipitate from the dialysed solution contained the whole of the activity in 3 % (inclusive of dye) of the original dry weight.

(2) Neutral red precipitates from gastric juice containing the whole of the original activity weighed, according to the p_H of precipitation, from 25 to 90 % (p_H 3 to 4·8) of the dry weight of the original preparation.

(3) The minimum efficient concentration of dye was found to be 0·1 %, and the optimum p_H for purification just above 3.

(4) The weight of inactive matter precipitated from Benger's trypsin by safranine was 17 % of the total.

Summary.

1. The observation of Marston and others is confirmed that the addition of safranine or neutral red to a solution of pepsin or trypsin leads to the separation of a flocculent precipitate. In the case of pepsin solutions, including gastric juice itself, the precipitate removed the peptic activity from solution. The supernatant liquors from the trypsin precipitates, on the other hand, lost no significant proportion of their activity.

2. The optimum conditions of concentration and p_H for the complete removal of pepsin from solution by this means have been determined and a method is described for the recovery of the precipitated pepsin.

3. It has been shown that azine dyes do not "poison" the catalytic activity of either pepsin or trypsin. Consequently there exists in solution under the experimental conditions no significant concentration of a specific protease-azine complex antagonistic to protein hydrolysis.

4. These results give no support to the view of Marston that the azine nucleus reacts specifically with a protease with the formation of a protease-azine complex. The formation of precipitates when azine dyes are added to protease preparations may not be used as an argument either for the piperazine structure of proteins or for any structural scheme of the mechanism of protease digestion.

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LXXII. NOTE ON THE ABSORPTION SPECTRUM OF VITAMIN A.

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It has been claimed repeatedly that vitamin A is characterised by a selective absorption in the ultra-violet region at about 320–328 $\mu\mu$ [Takahashi *et al.* 1925; Morton and Heilbron, 1928]. We have subjected this claim to the experimental test by preparing dehydroergosterol, a sterol with four double bonds, from ergosterol [Windaus and Linsert, 1928]. Spectroscopic measurements by a photographic method [Bourdillon, Fischmann, Jenkins and Webster, 1929], kindly carried out by Mr R. G. C. Jenkins, showed that this substance possessed an intense absorption exactly in the same region as that claimed for vitamin A. The absorption curve agrees with that previously obtained by a photo-electric method [Windaus and Linsert, 1928].

The free sterol as well as its acetate, however, proved to be devoid of growth-promoting properties. Incidentally, we prepared the peroxide by the action of white light on dehydroergosterol in the presence of eosin [Windaus and Linsert, 1928] and found it equally inactive. For the biological tests the substances were administered in olive oil to rats on a vitamin A-free diet in doses from 0·001 mg. to 1 mg. *per diem*, vitamin D being supplied as irradiated ergosterol.

The fact that these substances also fail to give a blue colour with $AsCl_3$ (or $SbCl_3$) is significant in view of the assumed association of this colour reaction with vitamin A. It would seem therefore that selective ultra-violet absorption at 320–328 $\mu\mu$, at any rate by itself, cannot be taken as a criterion of vitamin A.

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LXXIII. THE NATURE OF THE VITAMIN A CONSTITUENT OF GREEN LEAVES.

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In spite of the fact that green leaves form one of the most potent sources of vitamin A, comparatively little work has been done on the isolation of the active substance from these leaves. The material from an ethereal extract of spinach or other green leaves has been added to a synthetic diet in order to supply vitamin A by various observers, amongst them Osborne and Mendel [1919, 1, 2] and Willimott and Wokes [1927]. In some experiments which were being carried out by E. M. Hume and H. H. Smith, the unsaponifiable matter from which the sterol had been separated was used to supply the vitamin A constituent of the diet, and it seemed desirable to obtain as much knowledge as possible about the nature of the extract used.

Relation of greenness to vitamin A.

The work of Osborne and Mendel [1919, 1, 2] and Steenbock and Boutwell [1919] had indicated an association of the fat-soluble vitamin with the green parts of plants. This view was examined by Coward and Drummond [1921], who reported that vitamin A was not synthesised by etiolated shoots but that green leaves were active in its formation. Wilson [1922], on the other hand, found that etiolated shoots if given in sufficient quantity could supply the fat-soluble vitamin and that this factor was therefore formed in the absence of light. Coward [1923] concluded that, although the presence of chlorophyll was unnecessary, the formation of the fat-soluble vitamins required the action of light though not of ultra-violet radiation, and later [1925] made the interesting observation that green leaves which in the autumn had turned yellow were more active than the original green leaves. Wilson's work has been confirmed by Coward [1927, 1, 2], Moore [1927] and Heller [1928] and there seems now to be conclusive evidence that, although the green shoots show far more vitamin A activity than the etiolated ones, the latter can supply the active factor if they are given in sufficient quantity. The potency of the shoots is much increased under the influence of light and it cannot be considered as quite certain whether the activity of the etiolated shoots is

due to some effect of germination or to the experimental difficulty of ensuring that no light whatever shall reach the seedlings. A similar difference has been described between the green and white leaves of cabbage. Coward and Drummond [1921] noted that green cabbage leaves were more potent than white leaves in promoting the growth of rats on a diet deficient in the fat-soluble vitamins and Hume [1921] made similar observations for guinea-pigs. Steenbock and Sell [1922] reported that the green inside leaves of cabbages which had failed to head properly contained ten times as much pigment as white leaves on the inside of good heads and were far richer in what was then known as vitamin A, but at the same time they thought that more than the minimum demonstrable amounts were present in white cabbage.

Since the very active green leaves, and the inactive or very feebly active white leaves of cabbage may be obtained at the same time from the same plants, the unsaponifiable matter obtained from the lipoid extracted from white and green leaves respectively seemed very interesting material for investigation and we set out therefore to prepare unsaponifiable matter from both sets of leaves, to examine its chemical nature and to test it biologically for vitamin A activity.

In the present investigation a preliminary study of the white and green leaves of the large cattle cabbage led to the conclusion that three to four times as much unsaponifiable matter could be extracted from a given weight of green as from an equal weight of white cabbage. Not only was more lipoid matter extracted by light petroleum from the dried leaves, but the proportion of unsaponifiable matter derived from it was higher. It was just possible that the results of previous workers might be explained by the fact that equal weights of green and white cabbage contained very different amounts of unsaponifiable matter, although the nature of the unsaponifiable matter was identical.

The method of biological experiment.

In an investigation such as the present one, it is clear that the ultimate criterion for the vitamin A value of the materials studied must be the biological test, although many writers have found the latter far from satisfactory. In carrying out the series of tests described which has extended over about a year, a preliminary depletion period was always used and the aim at first was to obtain from the rats, on various doses of the materials to be tested, growth performances which should be comparable. As time went on, however, information accumulated as to the exact size of the minimal dose on which life could be sustained. It was then found that a better comparison could be made by ascertaining the dose which could just sustain life over a measured period and that which failed to do so. In this way it was possible to say, for instance, that the effective dose of one sample of carotene lay between 0·005 and 0·001 mg., while that of another lay between 0·01 and 0·005 mg. In working with doses so far sub-optimal the difficulty is encountered that

the number of animals which fail to recover when dosage begins after the depletion period is increased; for, when the animal is in a precarious state, the dose needed for recovery is often in excess of that just needed for maintenance. Also there is frequently a latent period of several days before the dose begins to act, in the course of which the animal may become very much worse and it is impossible then to tell whether the dose should be increased or not. The dose may be increased without there being a real necessity to do so or failure to increase it may bring about the loss of a specially prepared animal.

In the last series of experiments (spinach-carotene group) about to be described the results were very much impaired by the frequent occurrence in the animals of haematuria and of septic glands in the neck. Several fulminating cases of corneal ulcer also occurred about this time: it might be that the use of minimal doses favoured the development of the conditions; such was found to be the case independently by McCarrison [1927] and Fujimaki [1926], particularly in favouring the development of stone; but it seems more likely that, since in some cases the condition developed before dosage had begun, *i.e.* somewhere about the 7th week of deficient diet, it was also due to the implantation upon the deficient animals of an infection not previously observed among the experimental animals in this laboratory although frequently described by other workers [Macy *et al.*, 1927].

Where possible an attempt was always made to cure a rat which failed during the experimental treatment, by giving cod-liver oil or other material rich in vitamin A; when the attempt succeeded a positive control was thus supplied but when it did not succeed the failure was not significant.

The diet with addition of a source of vitamin B complex was as follows:

Heated caseinogen	300 parts
Wheat starch	750 ,,
Irradiated hardened cotton-seed oil ...	225 ,,
Salt mixture [Hume and Smith, 1928]...	75 ,,
Lemon juice	75 ,,
Distilled water	900 ,,

From 10 to 25 g. per head per day were eaten, 14 g. being a usual amount. The fractions to be tested for vitamin A were made up in the cotton-seed oil and administered separately with a pipette.

In previous experiments in which estimations of vitamin A have been made by Hume and Smith [1928], marmite has been relied upon as the source of the vitamin B complex, used in such an amount that an average daily consumption for a rat would be about 0·5 g. About the time when the present series of experiments was begun (July 1928) a number of cases was encountered where rats failed to respond by growth to doses of vitamin A believed to be adequate. These cases remained puzzling until one rat also developed skin lesions closely resembling those found in rats on a diet deficient in vitamin B_2 [Goldberger and Lillie, 1926; Chick and Roscoe, 1927]. Dried yeast auto-

claved at 120° for 5 hours, in which the antineuritic factor is almost completely destroyed while vitamin B_2 survives [Chick and Roscoe, 1927], was kindly supplied by Dr Chick and administered in amounts of 0·5 g. daily. Growth was restored and the dermatitis healed; the conclusion was drawn that marmite could not any longer be relied on as a constant source of supply of vitamin B_2. In the present series of experiments, marmite (75 parts in the above diet mixture) was at first retained and supplemented with 0·5 g. of the autoclaved dried yeast to every 10 g. of the wet diet. The animals used in the experiments, the results of which are given in Table I, received the vitamin B in this way. The animals used in the experiments, the results of which are given in subsequent tables, received no marmite in their diet. In those used in Table II 6 % of dried yeast from the Pharmaco Chemical Products Co. Ltd. was added to the wet diet, and in the final experiments on carotene this amount was increased to 10 or 12 %. The higher amounts appeared to give a more satisfactory result and also made it possible to rely more confidently upon the condition of the eyes as an index of vitamin A deficiency.

Preparation of lipoidal material from cabbage.

The method of preparing the material has already been described [Clenshaw and Smedley-MacLean, 1929]. The preliminary dipping of the leaves in boiling water, which is there described as rendering the leaves more brittle and more readily pulverisable, was used in preparing most of the material used for this investigation. It has however the disadvantage of setting free the chlorophyll so that this is also extracted in the light petroleum solution. If the leaves are dried at the ordinary temperature without the preliminary dipping in hot water, the light petroleum extract is yellow and is practically free from chlorophyll [Arnaud, 1885]. We have since found that a preliminary drying *in vacuo* before drying in the hot room also liberates the chlorophyll, but if the leaves are air-dried at a temperature of 37° the petroleum extract is yellow and appears to be free from chlorophyll. In nearly all the material used in this investigation, the leaves were subjected to the preliminary dipping in hot water, the petroleum solutions were deep green in colour and the lipoidal material contained chlorophyll. Treated in this way, 715 g. of dried white cabbage leaves yielded 7·58 g. of light petroleum-soluble material, whilst from 1300 g. of dried green cabbage leaves 25·83 g. of lipoidal material were extracted.

The material obtained from the green leaves was first tested for its activity in vitamin A. The medium chosen to dissolve the given extract was the same hardened cotton-seed oil as was used in the basal diet. In this preliminary experiment one set of doses was also given in paraffin oil in order to see if these were equally well absorbed. On the whole the effect seemed rather better with the hardened cotton-seed oil, especially in the case of the smaller doses and this medium was used in all later experiments. The results

are shown in Table I, which also contains the record of an experiment in which some dried green spinach leaves which had been several times extracted with light petroleum were extracted with acetone. Since xanthophyll is soluble in acetone and insoluble in light petroleum, this extract would contain the xanthophyll present in the leaves. No evidence of any vitamin A activity was found in this fraction, a result in agreement with that observed by Willimott and Moore [1927].

Table I. *Test of green cabbage and acetone-spinach extracts for vitamin A activity.*

Material tested	Dose mg.	No. of litter	No. of rat	Sex	No. of days maintenance	Growth in period of experiment g.	Notes
Green cabbage extract	5·0	1044	516	♀	28 +	43	— —
	,,	1045	518	♀	28 +	43	— —
(*a*) In cotton-seed oil	,,	,,	519	♂	28 +	66	— —
	2·5	1044	517	♀	25 +	15	Following larger dose for 15 days
	,,	1045	525	♀	25 +	15	Following larger dose for 14 days
	0·5	1044	515	♀	21 +	25	Following larger dose for 4 days
	,,	1045	523	♀	21 +	13	Following larger dose for 5 days
(*b*) In paraffin oil	5·0	1044	512	♀	28 +	43	— —
	,,	1045	524	♀	28 +	40	— —
	,,	,,	520	♂	28 +	61	— —
	2·5	1044	510	♀	18	3	Following acetone-spinach extract, died respiratory disease
	,,	,,	514	♀	25 +	10	Following larger dose for 11 days
	0·5	1045	526	♀	21 +	9	Following larger dose for 5 days
Acetone-spinach extract	10·0	1044	510	♀	8	2 −8	Rapidly deteriorating, cured by green cabbage extract
	,,	1045	527	♀	14	2 −5	Deteriorating, cured by other material
	,,	,,	521	♂	15	−7	Deteriorating, cured by other material

Note. Behaviour of rats when the material tested for vitamin A is supplied after a preliminary depletion period of 5–6 weeks. The growth or maintenance is for a measured period of 28 days though in one or two cases the period was arbitrarily shortened from lack of material or other cause. Where the symbol + fails to follow the days of maintenance it is indicated that a decline set in. Success or failure to maintain should be taken as the criterion and the amount of growth taken as a subsidiary comment.

Preparation of the unsaponifiable material.

This was prepared from the light petroleum-soluble material by adding to its ethereal solution at laboratory temperature an excess over the calculated amount of an alcoholic solution of sodium ethoxide, and allowing it to stand overnight. On diluting and extracting the solution with ether the chlorophyll remains in the aqueous alkaline solution.

From the green cabbage, 10·9 g. of unsaponifiable matter were obtained, a yield of 0·84 % of the dried leaves; the iodine value (Hübl) was 110·2. The white cabbage gave 1·78 g. of unsaponifiable matter, a yield of 0·25 % of the dried leaves; its iodine value was 60·5. The green leaves gave therefore three to four times as much unsaponifiable matter as an equal weight of white cabbage and the substance obtained from the green leaves was much more unsaturated than that prepared from the white cabbage.

Quantities of 1 g. of the unsaponifiable material obtained respectively from the green and white leaves were dissolved in a small quantity of ether and added to 25 g. of hardened cotton-seed oil. The ether was then blown off from the melted fat by a current of CO_2.

The results of the biological tests are given in Table II.

Table II. *Test of unsaponifiable matter from green and white cabbage for vitamin A activity.*

See note under Table I.

Material tested	Dose mg.	No. of litter	No. of rat	Sex	No. of days maintenance	Growth in period of experiment g.	Notes
Unsap. matter from green cabbage	1·5	1142	531	♂	28 +	49	
	1·0	,,	532	♀	28 +	31	
	,,	,,	535	♀	28 +	21	
	,,	1160	548	♂		—	Died at once
	0·5	1142	533	♀	28 +	22	Survived a pregnancy, litter lost
	,,	1160	543	♀	28 +	41	
	,,	,,	547	♂	28 +	48	
	0·25	,,	538	♀	28 +	23	Survived a pregnancy, litter lost
	,,	,,	541	♀	28 +	26	
	,,	,,	544	♀	28 +	28	
Unsap. matter from white cabbage	10·0	1160	539	♀	28 +	16	
	5·0	,,	542	♀	11	– 14	Cured by green unsap
	3·0	,,	540	♀	24	{21, – 10}	Maintained to 28th day by increasing dose to 10 mg
	,,	,,	545	♂	—	—	Died at once
	,,	,,	546	♂	14	{15, – 22}	Died
	1·5	1142	530	♂	16	{9, – 27}	Moribund, killed
	1·0	,,	534	♀	28 +	13	— —
	,,	,,	536	♀	15	25	Very ill, cured by green unsap.
	0·5		537		14	– 34	Pregnant, died
Green cabbage unsap. Fraction insol. in hot and cold alcohol. Sol. in light petroleum	1·2	1162	553	♂	28 +	56	
	0·6		555	♂	28 +	35	
	0·2		550	♀	28 +	15	
	,,		551	♀	28 +	23	
	0·03		554	♂	23 +	8	

FRACTIONATION OF THE UNSAPONIFIABLE MATERIAL.

Having established that the green leaves yielded an unsaponifiable matter at least ten times as potent in vitamin A activity as the white, the next step

was to fractionate this material and to find out how far this activity could be concentrated. The first method used was by extraction with different solvents.

Cold alcoholic extract. The unsaponifiable fraction was twice extracted with cold alcohol and, from the cold alcoholic extract of 5 g. of the original unsaponifiable matter, crystals of a substance melting at 135° separated on standing; these gave a positive Salkowski reaction and probably consisted of a sterol. A very small amount of red crystals was also separated from this extract; the filtrate from these was evaporated to dryness and yielded 2·3 g. of residue having an iodine value of 130·7.

Hot alcoholic extract. The substance insoluble in cold alcohol was three times extracted with hot 96 % alcohol. From this crystals separated melting from 63° to 68°. By subsequent recrystallisation from alcohol white crystals melting at 75° were obtained, and a substance melting up to 68°. A preliminary investigation of this fraction was described by Clenshaw and Smedley-MacLean [1929], who isolated a hydrocarbon which they regarded as identical with the hentriacontane, $C_{31}H_{64}$, M.P. 68°, which they had already isolated from spinach. Subsequently Channon and Chibnall [1929] published the results of an investigation on cabbage and described the isolation of a hydrocarbon melting at 62·7°–62·8° and of a ketone $C_{29}H_{58}O$ melting at 80·5–81°; these they identified respectively as nonacosane and di-*n*-tetradecyl ketone. The hydrocarbon isolated by Clenshaw and Smedley-MacLean from spinach melted sharply at 68–68·5° and was analysed and satisfactorily identified as the hydrocarbon $C_{31}H_{64}$. The substance which they obtained from cabbage, melting at 68°, was only separated in very small amount: it was not analysed, and for its identification a mixed melting-point with the hydrocarbon obtained from spinach was relied upon. In the present investigation, working with a green Christmas cabbage, we isolated two substances melting at 63° and 80° to 81° respectively and confirm the results published by Channon and Chibnall. We propose to examine again the large white summer cattle cabbage used in their investigation by Clenshaw and Smedley-MacLean in order to determine definitely whether the substance isolated by them was identical with the hentriacontane of spinach or was a mixture of the two substances identified by Channon and Chibnall.

Light petroleum extract. A small amount of blackish oily residue (iodine value, 173) remained which was practically insoluble in hot and cold alcohol: this was extracted with warm light petroleum (B.P. 40° to 60°) and from the solution red crystals of carotene separated. After several recrystallisations from light petroleum 0·018 g. crystals, which softened at 174° and melted at 178°, were obtained; they had an iodine value of 324. Very much less of the material from the white leaves was available than from the green: the whole of it was found to be soluble in alcohol and there was no blackish residue, corresponding to that obtained in working up green cabbage, insoluble in alcohol and soluble in light petroleum and rich in carotene. The quantity of

carotene present must have been so small as to have been completely dissolved in the alcoholic solutions.

Colour test with antimony trichloride. These various fractions were tested by the method of Carr and Price [1926] with the following results.

Green cabbage:

(1) Unsaponifiable matter	Blue-green colour
(2) Cold alcohol extract, 0·016 g. per cc.	Blue-green colour
(3) White substance from hot alcohol extract, 0·020 g. per cc.	Colourless
(4) Ether extract, insoluble in alcohol, 0·003 g. per cc.	Green colour
(5) Carotene crystals, 0·001 g. per cc.	Permanent blue colour
(6) Carotene crystals, 0·0001 g. per cc.	Permanent blue colour

White cabbage:

(7) Unsaponifiable matter	Blue-green colour, much less intense than (1)

The fact that solutions of carotene give a blue and not a green colour may be interpreted as showing that the yellow colour of carotene disappears and that it is therefore the yellow coloured carotene itself that enters into combination with the antimony trichloride solution. The green colour produced by the yellow unsaponifiable fractions which contain carotene must therefore be ascribed to a mixture of the blue compound formed from carotene and antimony trichloride with a yellow substance which remains yellow and is not changed by the antimony trichloride solution.

Preparation of carotene.

It appeared from the above results that the activity was associated with the carotene-containing fractions, and was certainly greatest in the most unsaturated fractions. It seemed desirable therefore to isolate carotene from the unsaponifiable matter and to determine whether the vitamin A active fraction was still associated with the carotene crystals. Three materials were selected, cabbage, spinach and carrots, and from each of these a small specimen of carotene was prepared.

From cabbage. 10·9 g. of the unsaponifiable matter from green cabbage were fractionated as described above. A very small amount of carotene was obtained from the fraction soluble in hot alcohol: the fraction insoluble in alcohol but soluble in ether deposited dark reddish crystals which were several times recrystallised and finally obtained softening at 174° and being completely melted at 178°. Examination of a dilute solution of this carotene in light petroleum solution showed the presence of two bands extending from 450 to 455 $\mu\mu$ and from 478 to 486 $\mu\mu$ respectively.

From spinach. When similarly treated 11·05 g. of the lipoidal matter from spinach yielded 5·09 g. of unsaponifiable matter (I.V. 108). In this case the carotene was separated by dissolving the unsaponifiable fraction in carbon disulphide and fractionally precipitating it with alcohol. The earlier precipitates contained most of the impurities; the later (I.V. 183) were rich in carotene and were redissolved in carbon disulphide, again fractionally precipitated with alcohol, and then several times recrystallised from ether. The iodine value was 352. After further recrystallisation, the material melted at 163–164° and had an iodine value of 338. This fall in value cannot be regarded as significant since very small quantities of material were used for the determinations.

Part of the material (I.V. 183) was recrystallised from ether, dissolved in light petroleum, shaken up with chalk and filtered through a layer of chalk. The solvent was then evaporated from the filtrate and the residue used for a biological test on one rat.

From carrots. Slices of carrots were dried at 37° and extracted with light petroleum. The residue from the petroleum extract (I.V. 142·3) was worked up similarly to the unsaponifiable matter from spinach. The reddish yellow crystals first obtained had an iodine value of 181°. By fractionally precipitating with alcohol from their carbon disulphide solution, crystals were obtained melting from 164° to 170° (I.V. 220). By further purification 22 mg. recrystallised carotene of iodine value 300 were obtained. Examined microscopically it consisted of characteristic red diamond-shaped crystals; at this stage it was used for the biological tests, and was compared with the cruder specimen of iodine value, 220.

Solutions (0·14 %) of all three specimens of carotene in hardened cottonseed oil gave a blue colour with the antimony trichloride reagent. A 0·07 % solution of the specimen from cabbage was also tested and gave a marked blue colour.

The evidence at present available as to the constitution of carotene, $C_{40}H_{56}$, indicates that it possesses 11 double bonds of which probably 8 are conjugated [Zechmeister and v. Cholnoky, 1928]. The iodine values for the specimens of carotene now examined varied between 300 and 350, corresponding to about 7 ethylenic linkages in the molecule. It seems unlikely that 22 halogen atoms would be added by interaction with the iodine reagent. It is known for instance that ethylenic linkages in the α–β position to an OH or COOH group do not react with the iodine reagent [Smedley-MacLean and Thomas, 1921], and the addition of so many halogen atoms would probably modify the reaction. The variations from 300 to 352 cannot be regarded as significant in view of the very small quantities of material which were used for these determinations; the experimental error was large, since only 2–3 mg. were available for the test, and might well be 10 % of the observed value.

The highest melting point of the various specimens of carotene was that from cabbage which softened at 174° and finally melted at 178°.

Table III. *Test of carotene for vitamin A activity.*

See note under Table I.

Material tested	Dose mg.	No. of litter	No. of rat	Sex	No. of days maintenance	Growth during period of experiment g.	Notes
Cabbage carotene:							
I.V. 330	0·01	1228	572	♀	28 +	40	— —
M.P. 174–178°	,,	1227	577	♂	28 +	76	— —
	0·005	,,	579	[illegible]	28	7	Moribund at end of experiment with septic bladder condition
	,,	,,	578	♂	28	60	— —
	0·003	1223	563	♂	28 +	25	— —
	,,	1225	601	♂	28 +	10	Following smaller dose, septic glands in neck
	0·002	1223	564	♂	28 +	16	— —
	,,	1275	601	♂	6	− 5	Following smaller dose, changed to larger dose
	0·001	1223	561	♀	24	{ 6 − 15 }	Maintained to end of period on 0·002 mg.
	,,	1228	574	♂	4	− 2	Cured by cod-liver oil
	,,	1275	604	♀	3	− 5	Died—septic uterus
	,,	,,	605	♀	14	5 − 4	Haematuria
	,,	,,	601	♂	6	− 5	Changed to larger dose
Carrot carotene:							
(*a*) I.V. 220	0·04	1272	596	♀	28 +	29	— —
M.P. 169°	0·02	1223	567	♂	28 +	7	Following dose mother-liquor
	,,	1272	600	♀	28 +	23	— —
	,,	,,	597	♂	28 +	29	— —
(*b*) Recryst. I.V. 300	0·01	1223	560	♀	28 +	31	— —
	,,	1228	573	♂	7	− 6	Fulminating corneal sepsis. Treated perhaps prematurely with other material—cured
	,,	1227	576	♂	28 +	36	— —
	0·005	1275	607	♀	10	− 4	Cured by cod-liver oil
	,,	,,	602	♂	16	—	Changed to 0·01 mg. with temporary improvement
	0·004	,,	608	♀	13	− 8	Cured by cod-liver oil
	0·002	1275	603	♂	12	− 8	Cured by cod-liver oil
	0·001	1228	575	♂	5	− 4	Cured by other material
	,,	1275	608	♀	13	− 8	Changed to 0·004 mg.
	,,	,,	607	♀	12	− 10	Changed to 0·005 mg.
	,,	,,	602	♂	7	− 8	Changed to 0·005 mg.
Spinach carotene:							
(*a*) I.V. 338 M.P. 164°	0·012	1300	611	♂	8	—	Haematuria, died, severe stone
	,,	,,	616	♂	28 +	36	Foetid urine
	0·008	,,	609	♀	28 +	15	Haematuria before beginning dosage
	,,	,,	610	♀	18	{ 4 − 13 }	Haematuria—died
(*b*) I.V. 352	0·010	,,	613	♂	28 +	51	Foetid urine—enlarged gland in neck
	,,	,,	614	♂	8	− 10	Haematuria before beginning dosage—died
	,,	,,	615	♂	25	{ 25 − 14	Haematuria—recovered with cod-liver oil
(*c*) After shaking solution with, and filtering through, chalk	0·006	1300	612	♂	28 +	27	— —

The separation of carotene from cabbage is certainly very incomplete, especially when, as in our separation, no stringent precautions were taken to guard against oxidation. The actual amount of crystals separated must represent only a small proportion of the carotene present in the original cabbage.

Figures are not available as to the minimum weight of fresh or dried cabbage which will maintain a rat on a vitamin-A deficient diet in a good state of health under the conditions of the experiment.

From the figures now given 11·17 kg. cabbage leaf gave 1300 g. dry material from which 25 g. lipoidal substance and 10·9 g. unsaponifiable matter were extracted. In our experiments 0·25 mg. of unsaponifiable matter was more than sufficient for the minimum dose to supply the vitamin A factor and must therefore contain the minimum dose of carotene, here determined as 0·003 to 0·005 mg. This would correspond to rather more than 2 % of carotene in the unsaponifiable matter. Channon and Chibnall [1929] estimate the amount of carotene in the acetone-ethereal extract of cabbage with which they worked as 0·86 % and as the unsaponifiable fraction would be only a portion of their total extract, these figures seem to be of a comparable order.

The biological results are given in Table III.

The specimen of carotene which melted at the highest temperature and was therefore presumably the most pure was that from cabbage which was active in a dose of 0·003 mg. *per diem*: in this group the division between the active doses which maintained life for 28 days with fair growth and those of 0·001 mg. which failed to do so was well brought out. The specimens of carotene from spinach and from carrots respectively were less pure, the melting point was lower and the positive dose required was appreciably higher, being in the neighbourhood of 0·01 mg. The animals were not in such good condition in the later groups used for these experiments and it is not therefore permissible to make the deduction that the purity of the carotene specimen is in inverse ratio to the dose required, but we can say that we found no indication that the activity of the dose diminished with increasing purity of the carotene. The residue from the mother-liquor from which we had separated the carotene crystals from cabbage was tested on three rats and gave negative results with doses of 0·005 and 0·02 mg., doses which were active in the case of the carotene crystals, so that there was a definite concentration of activity associated with the carotene crystals. The residue from which the carotene had been extracted with ether was fed to 5 rats and was inactive in doses up to 0·1 mg.

The material obtained by shaking up an ethereal solution of the spinach carotene in light petroleum with finely precipitated chalk and filtering through a layer of chalk, and evaporating the solvent, showed no diminution in activity; a dose of 0·006 mg. gave a good result but only one rat was available for this test (Table III, no. 612). It seems that the active substance, like the carotene, is not absorbed by chalk.

Discussion.

The results obtained with carotene are closely in agreement with those recently published by v. Euler *et al.* [1928, 1929] and by Moore [1929]. The association of vitamin A activity with the carotenoid pigments has been frequently referred to by different workers. The idea has been supported in a series of papers by Steenbock and Sell [1922], who however do not definitely identify the active factor as carotene: the association of the carotenoids with the active vitamin A substance was discussed by Rosenheim and Drummond [1920] but the identification of the vitamin A as carotene was definitely rejected by them and by Drummond, Channon and Coward [1925]. The latter observers isolated from carrots a specimen of carotene which after being recrystallised four times melted at 167·5°, and which they regarded as free from vitamin A activity, although the dosage given is not stated. More recently Dulière, Morton and Drummond [1929] have separated from carrots a specimen of carotene melting at 184·9° after recrystallising it several times from hexane and working under anaerobic conditions. This preparation was found to be almost inactive even in doses of 0·5 mg.

The fact that both specimens of carotene isolated by Drummond and his colleagues, melting respectively at 167° and 183·9°, were found to be inactive suggests that the inactivity of the purer specimen described in the more recent work is not due to the elimination of an active impurity although the possibility that Dulière, Morton and Drummond have isolated an inactive constituent from the crude carotene cannot be entirely excluded.

All the workers who find that carotene shows vitamin activity include fat in their basal diet, whereas Drummond and his co-workers give a fat-free diet. It is possible that in the latter case the pigment is not so well absorbed and it is interesting to note that Hart, Steenbock, Kletzien and Scott [1927] found that the antirachitic unsaponifiable constituent of cod-liver oil did not establish the calcium balance in a goat unless it was given in solution in a liquid fat such as corn oil, although the corn oil itself had no effect on assimilation[1]. On the other hand, Hume and Smith (unpublished experiment) have found that the unsaponifiable fraction from a light petroleum extract of spinach, freed from sterols, is an adequate source of vitamin A, when dissolved in liquid paraffin and added to a fat-free diet. The latter observation suggests that it may be some element of the unsaponifiable fraction of the fat in the diet, rather than the fat as fat, which is needed in conjunction with carotene, to produce the biological effect of vitamin A.

Evidence appears to be accumulating tending to show that more than one substance can function as vitamin A. Differences in the stability of the vitamin A derived from plant and animal sources have been described [Sherman, Quinn, Day and Miller, 1928], and it seems difficult to accept as

[1] Since writing the above, attention has been drawn to the influence of fat in the diet by Burr and Burr [1929] and McAmis, Anderson and Mendel [1929].

identical the highly coloured crystals which are associated with the vitamin A activity in plants with the much paler substances derived from animal oils. The recent publication of v. Euler, v. Euler and Karrer [1929], which records the inactivity of α-crocetin and the activity of its dihydro-derivative, furnishes evidence in support of the view that the property of vitamin A activity is to be regarded rather as belonging to a special grouping of atoms which may be common to several individual molecules than to a definite molecule.

Dulière, Morton and Drummond have laid stress on the appearance of an absorption band 608–612 $\mu\mu$ in the reaction product of antimony trichloride and specimens of the unsaponifiable matter from cod-liver oil which are very active in vitamin A and have drawn the inference that this band is associated with the actual vitamin A. The absorption spectrum of the blue compound produced when carotene reacts with the same reagent does not show this particular band [v. Euler *et al.*, 1928], though it is suggested that the difference may be due to the presence or absence of an oily medium. If the same vitamin A which produces this band be indeed a contamination in the carotene crystals, as Dulière *et al.* suggest, it is difficult to see why the characteristic 608–612 $\mu\mu$ band does not appear in the absorption spectrum of the carotene compound. Since the carotene crystals are as potent in vitamin A activity as any unsaponifiable fraction from animal oils yet investigated, one would expect the 608–612 $\mu\mu$ band to be as strongly marked in the spectrum of the carotene antimony trichloride compound. If the vitamin A of animal oils be indeed responsible for the production of the band, its absence when the very active carotene crystals are used would furnish additional evidence that the active substance in the cod-liver oil is not identical with that in the active carotene crystals.

Summary.

(1) The vitamin A activity of the unsaponifiable fraction from white cabbage is very small compared with that of the corresponding fraction derived from green leaves; this fraction, however, is active if given in sufficient amount. The respective minimal doses are about 10 and 0·25 mg.

(2) The vitamin A substance of green spinach and cabbage leaves and of carrots is contained in the most highly unsaturated fraction of the unsaponifiable matter and, as far as the process of purification here employed extends, remains associated with the carotene crystals.

(3) The carotene crystals obtained from cabbage softened at 174° and melted at 178°, those from spinach at 163–4° and from carrots at 164–9°. The vitamin A activity is certainly not diminished in the specimen of higher melting point and of therefore presumably greater purity: in this specimen the active dose lies between 0·002 and 0·005 mg. No claim to a great degree of purity in the specimens of carotene separated is made, since the work carried out was on too small a scale to admit of sufficient recrystallisations to ensure the separation of all impurities.

(4) It is possible that the crystals of carotene may themselves be homogeneous and active or that they may consist of two or more closely related substances only one of which possesses vitamin A activity.

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LXXIV. THE DETERMINATION OF CHLORIDE IN ANIMAL TISSUES.

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During the course of an investigation of salt-cured meat, it became necessary to find a highly accurate method for the estimation of chloride in both salt-cured and fresh meat. It was essential that the method should be applicable to samples as large as 50 g., and that it should be simple and reliable. In order to determine the accuracy of various methods, estimations were carried out of the chloride content of (*a*) fresh meat, and (*b*) an equal weight of the same sample of fresh meat to which had been added a known amount of sodium chloride. Lean pork was minced three times and well mixed. 50 g. portions were placed in well-stoppered glass bottles with wide necks. The contents of half the bottles were analysed in order to determine the chloride content of the fresh meat. Known amounts of sodium chloride were added to the remaining bottles, which were kept at 0° for several days to allow the salt to react with the tissue. The chloride content of the salted meat was then determined.

There are two stages in the chloride analysis of meat. First, the chloride must be extracted from the tissues with as little protein as possible, and secondly the chloride content of the extract must be determined. For the second stage, the Volhard method was used in this investigation. The first stage, namely extraction, is frequently carried out after the organic matter has been charred by heat. This method gives low values for the chloride content because a certain amount of chloride is invariably lost by volatilisation. The results obtained by this method were lower than those obtained by any of the other methods investigated (see Table I). The incineration method has been modified by Weitzel [1920]. He mixed the substance to be analysed with calcium oxide before incineration. There is no doubt that this is an improvement, but even the modified method in our hands gave low results for the chloride of meat (see Table II).

As incineration was found to be unsatisfactory, an attempt was made to find other ways of obtaining the chloride in solution with minimal amounts of protein. It seemed probable that the most promising method would be to use a boiling aqueous salt solution which would extract the chloride and at

the same time coagulate the proteins. Solutions of sodium sulphate and of iron alum were used but gave results which were either too low or inconsistent. Distilled water alone, however, proved to be an excellent reagent for extraction.

Table I. *Typical results obtained for chloride estimation of meat.*

Various methods of extraction were used and in each case the extract was analysed by Volhard's method. The same sample of pork was used throughout.

Method of extracting chloride from meat	Chloride (expressed as g. NaCl) found in 50 g. of lean pork	Chloride (expressed as g. NaCl) found in 50 g. of lean pork to which had been added 2 g. NaCl	NaCl recovered g.	Percentage recovery
Boiled with distilled water	0·055	2·062	2·007	100·4
Boiled with 20 % sodium sulphate	0·053	2·046	1·993	99·7
Boiled with 20 % sodium sulphate plus iron alum	0·056	2·036	1·980	99·0
Boiled with distilled water plus iron alum	0·046	2·002	1·956	97·8
Incinerated at a dull red heat	0·041	1·982	1·941	97·1

Table II.

The same sample of pork was used throughout. In each case the results are the average of two determinations.

NaCl added to 50 g. of pork g.	Extracted with boiling distilled water: NaCl found g.	NaCl recovered g.	Percentage recovery	Incinerated with calcium oxide (Weitzel's method): NaCl found g.	NaCl recovered g.	Percentage recovered
Nil	0·054	—	—	0·051	—	—
1	1·044	0·990	99·0	1·026	0·975	97·5
2	2·051	1·997	99·9	2·003	1·952	97·6

The following procedure was finally adopted. 50 g. of finely minced meat are placed in a 300 cc. beaker and covered with distilled water. The beaker is placed in a boiling water-bath, and the contents are stirred in order to prevent the formation of large lumps during the coagulation of the protein. The beaker is then heated over a Bunsen burner until the contents boil. The precipitate is allowed to settle and the clear fluid filtered into a flask. The residue is again boiled with distilled water, and the solution decanted and filtered into the collecting flask. In the case of fresh meat this process is repeated 5 or 6 times and the filtrate is transferred to a 1000 cc. standard flask. With salted meat it is necessary to boil the residue 10 or 12 times with distilled water. The filtered solution in this case is made up to 2000 cc. Measured volumes of this solution (with fresh meat and with very low concentrations of sodium chloride the solution must be concentrated by evaporating 250 cc. to about 10 cc.) are then placed in boiling-tubes with an excess of silver nitrate. A volume of concentrated nitric acid equal to the volume of the mixture is added, and the tubes are heated in a boiling water-bath for

an hour and a half to destroy the proteins. When cool, distilled water is added to dilute the strongly acid solution. The silver chloride is filtered off and washed, and the excess of silver nitrate is estimated by the Volhard method.

A typical series of results is given in Table III.

Table III.

The same sample of pork was used throughout.

Weight of NaCl added g.	Total NaCl in 50 g. meat g.	NaCl recovered g.	Percentage recovery
Nil	0·053, 0·051, 0·048 } average 0·051		
0·2	0·246	0·195	97·5
0·2	0·248	0·197	98·5
0·5	0·552	0·501	100·2
0·5	0·553	0·502	100·4
1·0	1·050	0·999	99·9
1·0	1·055	1·004	100·4
2·0	2·051	2·000	100·0
2·0	2·041	1·990	99·5

These results show that sodium chloride which has been added to meat can be estimated quantitatively by this method. Although there is complete recovery of added sodium chloride, it does not necessarily follow that this method accurately estimates the chloride originally present in the meat. In order to test this point, the following experiments were carried out. After extracting the chloride by the method described above, the residues were cautiously incinerated at dull red heat. The charcoal formed was extracted with water and then ashed. No trace of chloride was detected in the charcoal extract or ash. Even when the extracted residues from 300 g. of fresh meat were bulked together for this purpose, no chloride could be detected. This indicates that the chloride is completely extracted from meat by this method. The question then arises as to whether the method is equally reliable when applied to commercially salt-cured meats, which contain saltpetre and often sugar in addition to sodium chloride. Estimations carried out on similar samples of such material gave remarkably concordant results, considering the difficulties of sampling. For example, six estimations of a sample of minced ham gave the following results for the concentration of chloride expressed as sodium chloride: 8·04 %, 8·02 %, 7·96 %, 7·88 %, 8·04 % and 8·02 %. Four estimations of a sample of cooked ham gave the following results: 4·94 %, 4·84 %, 4·84 %, 4·84 %. After extraction and incineration, the coagulated residue showed no trace of chloride.

An alternative method of estimating small quantities of chloride in biologica fluids has been put forward recently by Christy and Robson [1928]. They add $N/29·25$ silver nitrate to the solution in the presence of nitric acid; the silver chloride is filtered off, and the excess of silver nitrate in the filtrate is titrated against $N/29·25$ potassium iodide in the presence of potassium

bi-iodate and soluble starch. Silver iodide is precipitated and, when the reaction is complete, further addition of potassium iodide results in the formation of iodine. According to Christy and Robson, the slightest excess of potassium iodide results in the formation of the well-known blue colour of starch iodide. I have found, however, that the end-point is obscured by the appearance of a yellow colour which changes the clear blue to a muddy green. Moreover, I found that the end-point is not reached until a considerable excess of KI has been added.

In order to test the method, I carried out preliminary analyses on a solution containing a known amount of sodium chloride. I followed Christy and Robson's procedure except that I used $M/100$ potassium bi-iodate, as it is not clear what is meant by $N/100$ potassium bi-iodate. In every case the end-point of the titration was greenish and not well defined, and the results were 5–10 % too low. A large number of control titrations were then carried out in order to discover the cause of these grave discrepancies. Using 10 cc. of $N/29\cdot25$ silver nitrate, no end-point was obtained until more than 11 cc. of $N/29\cdot25$ potassium iodide had been added. In Christy and Robson's method 1 cc. of $N/100$ potassium bi-iodate is used, but I found that the

Table IV.

$N/29\cdot25$ silver nitrate cc.	$M/100$ potassium bi-iodate cc.	$2N$ nitric acid cc.	1 % soluble starch cc.	Water added cc.	Potassium iodide cc.
10	1·00	10	2	90	11·8
10	0·50	10	2	90	10·8
10	0·20	10	2	90	10·45
10	0·10	10	2	90	10·31
10	0·05	10	2	90	10·20
10	1·00*	10	2	90	10·92
10	0·50*	10	2	90	10·63
10	0·20*	10	2	90	10·26
10	0·10*	10	2	90	10·20
10	0·05*	10	2	90	10·30
10	1·0	10	1	90	11·81
10	1·0	5	1	95	11·54
10	1·0	2	1	98	11·26
10	1·0	1	1	99	10·92
10	1·0	0·5	1	99·5	10·80
10	1·0	0·2	1	99·8	10·36
10	1·0	0·1	1	99·9	10·32
10	1·0	10	2	90	12·02
5	1·0	10	2	90	6·47
2	1·0	10	2	90	2·91
1	1·0	10	2	90	1·66
0·5	1·0	10	2	90	0·84
Nil	1·0	10	2	90	0·05
10	1·0	10	1	90	11·72
10	1·0	10	1	80	11·90
10	1·0	10	1	60	11·87
10	1·0	10	1	40	11·78
10	1·0	10	1	20	11·80
10	1·0	10	1	10	11·92
10	1·0	10	1	Nil	11·93
10	0·10	10	1	Nil	10·30

* KIO_3.

results agreed more and more closely with the expected figure as the concentration of bi-iodate was decreased until, in the presence of 0·10 cc., only 10·2 cc. of potassium iodide were needed for 10 cc. of silver nitrate. The end-point became progressively sharper as the concentration of bi-iodate was decreased, the muddy green colour being gradually replaced by a clear blue colour. With less than 0·05 cc. of $M/100$ potassium bi-iodate the end-point was indefinite because the blue colour was too pale. When potassium iodate was used instead of the bi-iodate, similar results were obtained. Other control titrations involved variations in the amounts of silver nitrate, nitric acid and water (see Table IV). The titration values of potassium iodide necessary to give an end-point are given in the last column.

On titrating in the absence of starch and filtering off the silver iodide formed, the filtrates obtained were yellow to orange-brown in colour. It was naturally suspected that the yellow colour was due to the formation of iodine, but this hypothesis at first appeared untenable because of the non-appearance of a blue colour with starch. Further experiments, however, indicated that iodine was actually present. It could be extracted by means of chloroform or obtained as a sublimate in the condenser on vacuum distillation. Since the yellow colour was due to iodine, it may be asked why no blue colour was obtained with starch. The answer to this question is the usually neglected fact that a solution of iodine in water is incapable of giving a blue colour with starch, except in the presence of a soluble iodide [Treadwell, 1921].

Thus, when solid iodine is allowed to remain in contact with a solution containing soluble starch, nitric acid and potassium iodate (or bi-iodate), no blue colour appears. Under these conditions, even if HI is formed, it immediately reacts with the iodate and nitric acid to give free iodine:

$$5HI + KIO_3 + HNO_3 = 3I_2 + 3H_2O + KNO_3.$$

The fluid gradually becomes yellow and then orange-brown, and iodine can be extracted by shaking with chloroform. In all respects this fluid is similar to the yellow or orange-brown fluid previously described as occurring in the control titrations after filtering off the silver iodide. In the control titrations potassium iodide is run into a solution containing silver nitrate, potassium bi-iodate (or iodate), nitric acid and soluble starch. The blue colour of the starch iodide does not appear at the point when all the silver has been precipitated (as silver iodide) because further additions of potassium iodide react with the iodate or bi-iodate and nitric acid to give free iodine. This iodine gives no blue colour with starch because there is no soluble iodide present. From theoretical considerations we should not expect that a blue colour would be formed until enough potassium iodide has been added to decompose all the potassium bi-iodate present in the solution. In actual practice, however, it has been found that when starch is present the blue colour is formed before the theoretical amount of potassium iodide has all been added (see Table IV). This appears to be due to the fact that starch

iodide is a relatively stable compound and when once it has been formed it is difficult to decompose. This point is illustrated by the following experiment. When a large excess of potassium iodate (or bi-iodate) is added to a trace of potassium iodide in a solution of nitric acid, iodine is formed and all the potassium iodide is decomposed. On adding starch, no blue colour is produced. If however starch is added to the potassium iodide and nitric acid before the iodate or bi-iodate, a deep blue colour is immediately produced.

Christy and Robson's method is therefore subject to two sources of error. In the first place a considerable amount of free iodine may be present before the blue end-point is reached, and in the second place the excess of potassium iodide required varies with the concentrations of the reacting substances. It is conceivable that it might be used as an empirical method, but only under rigidly standardised conditions. This fact was emphasised by Bang [1913], who devised the procedure which forms the basis of Christy and Robson's method.

SUMMARY.

(1) A method is described for the complete extraction of chloride from animal tissues.

(2) Chloride in such extracts was estimated with a high degree of accuracy by means of a modification of Volhard's method.

(3) A criticism is given of Christy and Robson's method for the estimation of chloride in biological fluids.

I gladly take this opportunity of thanking Sir William B. Hardy, F.R.S., for the interest he has taken in this work. I also wish to thank Mr David Gatherum for carrying out many of the analyses recorded in this paper.

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LXXV. THE SALT EFFECT ON THE INDUCTION PERIOD IN THE FERMENTATION BY DRIED YEAST.

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The effect of a number of substances on the period of induction during which no fermentation is observed with the mixture of sugar solution and zymase preparations has been observed by many authors. Meyerhof [1918] pointed out that with maceration extract this period was completely abolished in the presence of even a small amount of hexosediphosphate. This was tested first by Harden and Henley [1920] with zymin and later by Mayer [1927] with maceration extract. Raymond and Levene [1928] found that not only natural hexosephosphates (hexosediphosphate, Robison's and Neuberg's monophosphates) but also synthetic phosphates (3-glucose-, 1-fructose-, 3-fructose- and dihydroxyacetone-phosphates) had a marked effect in reducing the period of induction with zymin in large volumes of sugar solution, although hexosediphosphate greatly exceeded all the others.

Neuberg [1918] pointed out a remarkable effect of a number of aldehydes and other hydrogen acceptors in accelerating fermentation with maceration extract. These observations were confirmed by Harden and Henley [1920 and 1921, 1] with zymin.

Recently Harden [1925], and subsequently Harden and Macfarlane [1928], made the interesting observation that the period of induction in fermentation by zymin in large volumes of sugar solution was appreciably reduced by the addition of various inorganic and organic salts.

In the present paper the effect of inorganic and organic salts on the period of induction in the fermentation by dried yeast (prepared from the bottom type beer yeast in Japan) in the presence of phosphate has been studied in order to ascertain which salt would be more potent in reducing the period of induction, and what influence would be exerted on the period of induction when a mixture of salts is employed.

Methods and results.

The apparatus described by Harden, Thompson and Young [1910] was used. The fermentations were carried out at 25° by a suitable amount of dried yeast (about 0·8 g.) with 20 cc. of the various salt solutions containing

2 g. glucose, Na_2HPO_4 (0·04 M) and 0·2 cc. toluene, after saturation with CO_2. The amount of CO_2 evolved was observed at intervals of 10 minutes. The p_H values of the mixtures after saturation with CO_2 were determined colorimetrically in the same manner as was described by Biilmann and Katagiri [1927]. The p_H values were found to be 6·3, and no difference in p_H on the addition of the salts could be detected.

For the period of induction, the intersecting point of the two tangents to the curves of normal fermentation and of the initial slow fermentation was chosen as shown in Fig. 1.

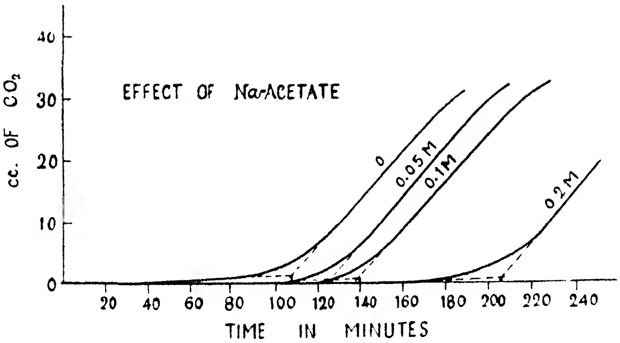

Fig. 1.

In order to allow for variation in the period of induction according to the sample of dried yeast, a fermentation was always carried out in the presence of 0·04 M phosphate without any other salt. The standard induction period under these circumstances was taken as 100 minutes. When the observed period had not greatly differed from this, the ratio of the two numbers was found and used as a factor to correct all the observations of the series to the standard.

When the sample of dried yeast had a considerably different fermentative power, the amount of dried yeast taken in each series was determined by a preliminary test so as to obtain nearly the same standard induction period (100 minutes). This amount of dried yeast varied from 0·75 to 1·0 g.

In order to test the validity of this method a series of experiments was made with 0·75, 0·8 and 1·0 g. of very different samples of dried yeast (Table I, Exps. 1, 2 and 3).

Table I.

Concentration of Na acetate (M)	Induction period (minutes) Exp. 1	Exp. 2	Exp. 3	Relative induction period Exp. 1	Exp. 2	Exp. 3
0	73	90	108	100	100	100
0·025	81	100	123	111	111	114
0·05	93	120	128	128	133	119
0·1	95	127	139	130	141	129

It will be seen that the relative values agree well, although the actual values were, in each case, considerably different.

The results of the experiments which are given in Tables I–VI show the average values of more than two duplicated observations. No remarkable difference in the rate of evolution of CO_2 during the rapid fermentation, in each series of experiments, could be detected. However, when still higher concentrations of the salts than the maximum concentration shown in the tables were employed, a reduced rate of fermentation was always observed, as well as a longer period of induction, as was expected from the observations of Harden and Henley [1921, 2].

Effect of sodium salts.

(*a*) *Organic salts.* Table II (A) shows the effect of the salts of fatty monobasic and hydroxy-acids.

Table II (A). *Relative induction period with monobasic acids.*

Concentration of Na salt (M) ...	0·025	0·0375	0·05	0·075	0·1	0·2
Formate	98	—	96	—	105	147
Acetate	114	—	119	–	129	178
Propionate	112	123	138	207	>250	—
Butyrate	104	—	121	—	>180	—
*iso*Butyrate	94	—	115	—	136	—
Glycollate	181	—	196	—	>260	—
Lactate	116	—	150	–	215	—
β-Hydroxybutyrate	102	—	114	–	186	—

It will be seen that the period of induction is prolonged by the addition of these salts and the effect is more potent as the concentration of the salts increases, except formate and *iso*butyrate; with these a slightly reduced period is observed at lower concentrations. However, the observed effects are not the same: among the simple fatty acids formate has the least, while propionate has the greatest effect on the period of induction.

The effect of the hydroxy-acids is in each case greater than that of the corresponding fatty acid. The order of the potency of the effect of hydroxy-acids is inversely proportional to their molecular weights, while in the case of fatty acids no such simple relation could be observed.

Table II (B). *Relative induction period with polybasic acids.*

Concentration of Na salt (M) ...	0·00625	0·0125	0·025	0·05
Succinate	118	166	198	>290
Fumarate	119	182	>280	—
Malate	128	153	>230	—
Tartrate	155	200	>280	—
Citrate	270	>300	—	—

It will be seen in Table II (B) that the effect of succinate, fumarate, malate, tartrate and citrate is in each case greater as the concentration increases. The shortest induction is observed with succinate and the longest with citrate. Fumarate, malate and tartrate produce nearly equal effects.

The hydroxy-acids are more potent than the corresponding fatty acids, and the di- and tri-basic acids have a greater effect than the monobasic acids

in prolonging the induction with dried yeast in presence of 0·04 *M* sodium phosphate and sugar. This variation in the magnitude of the effect on the induction period is probably due to the specific nature of the salts, that is, to the nature of their ions.

(*b*) *Inorganic salts.* In order to ascertain the effect of inorganic anions, experiments were carried out with NaCl, NaI, $NaHCO_3$, Na_2SO_4. The results are given in Table III.

Table III. *Relative induction period.*

Concentration of salt (*M*) ...	0·00625	0·0125	0·025	0·05	0·1	0·2
NaCl	97	100	109	140	272	>300
NaI	136	>215	—	—	—	—
$NaHCO_3$	123	134	138	213	>310	—
Na_2SO_4	114	124	131	190	>250	—

It will be seen that these salts produce an effect similar to that of the Na salts of organic acids. The period of induction is again prolonged by the addition of these salts, and the effect is greater as the concentration of these salts increases. The only exception is NaCl, with which a slightly reduced induction period is observed at the lowest concentration.

When the relative induction periods are plotted against the concentration of Na^+ of the salts added, the curves in Fig. 2 show the relative potency of the effect of their anions on the period of induction.

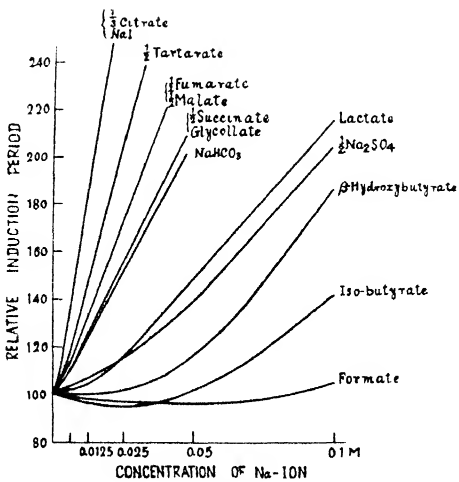

Fig. 2.

It will be seen that the iodide ion produces the greatest, and the formate ion the least prolongation of the period of induction.

Effect of K, Mg, NH_4 and Ca salts.

In order to investigate the effect of cations and to obtain further evidence of the specific effect of anions on the period of induction, experiments were made with various salts (Table IV).

Table IV. *Relative induction period.*

Concentration of salt (M) ...	0·00625	0·0125	0·025	0·05	0·1	0·2
KCl	88	90	82	89	110	177*
KI	109	104	177	>220	—	—
KBr	64	38	34	30	26	48
KNO_3	110	109	133	150	207*	>250
K_2SO_4	67	47	41	35	48*	84*
$MgCl_2$	—	85	81	73	91	213*
$MgSO_4$	—	67	47	29	25	37*
$CaCl_2$	—	102	110	>210	—	—
NH_4Cl	89	66	58	60	61	106
$(NH_4)_2SO_4$	15	19	19	23	32*	59*
NH_4 acetate	22	17	13	14	14	13
NH_4 lactate	32	22	15	15	14	22
NH_4 succinate	22	20	19	27	32	44*

* Represents the point at which a noticeably diminished fermentation rate is observed.

It will be seen that KI and KNO_3 produce an effect very similar to that of NaI; no reduction in the period of induction is observed at any concentration of these salts and the period is prolonged as their concentration increases. However, with KCl a reduced period is observed at the concentrations of 0·05–0·00625 M; the period of induction is gradually reduced until the concentration reaches 0·025 M and is then prolonged as the concentration increases. K_2SO_4 is more effective and KBr still more effective than KCl in reducing the period of induction. With these two salts the period is at first very rapidly, then very slowly reduced until the concentration reaches the optimum, after which the period is gradually prolonged as the concentration of these salts increases.

With both magnesium and ammonium salts (chloride and sulphate) a shorter period of induction is observed when these are compared with the corresponding potassium salts, and ammonium salts are more effective than magnesium salts in reducing the period. Among all the salts, $CaCl_2$ produces the greatest effect in prolonging the period of induction.

These observations show on one hand the specific effect of cations, and on the other that of anions. It is clearly seen, for example, in Fig. 3 in which the relative induction periods are plotted against the concentration of chloride ion, that the calcium ion produces the longest and the ammonium ion the shortest induction period. $\frac{1}{2}Ca^{++} > Na^+ > K^+ > \frac{1}{2}Mg^{++} > NH_4^+$.

Tables II, III and IV show that this order does not differ greatly when the sulphates are compared, and organic NH_4 salts (acetate, lactate and succinate) are much more effective than Na salts in reducing the period of induction.

The interpretation of this remarkable effect of ammonium salts is not very clear, but it is interesting to note that no specific effect of ammonium ion, as compared with sodium ion, is observed on the rate of normal fermentation of English living yeast [Katagiri, 1926].

As regards the specific effect of anions, it follows from the results in

Tables III and IV that the iodide ion produces the longest and the bromide ion the shortest induction period, the order of the anions being as shown below:

$$NaI > NaHCO_3 > NaCl > \tfrac{1}{2}Na_2SO_4,$$
$$KI > KNO_3 > KCl > \tfrac{1}{2}K_2SO_4 > KBr.$$
$$MgCl_2 > MgSO_4.$$
$$NH_4Cl > \tfrac{1}{2}(NH_4)_2SO_4.$$

No direct comparison of these results with those of Harden and Macfarlane [1928] is possible, since the conditions of the experiment were different from theirs, dried yeast being used instead of zymin and an addition of 0·04 *M* phosphate being made. However, the results obtained with KCl, NH_4Cl, K_2SO_4 and $MgSO_4$ coincide with their observations that the induction period is reduced from 60 to 20 minutes in the presence of these salts, although the optimum concentrations of these salts are not quite identical.

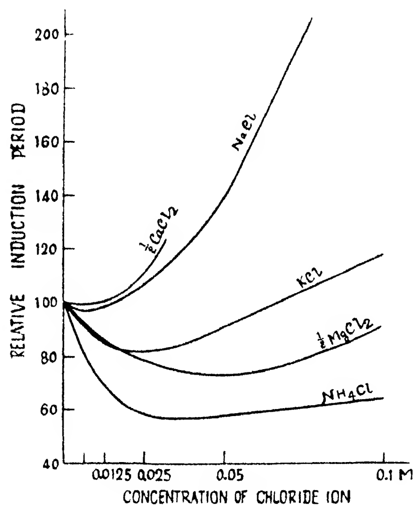

Fig. 3.

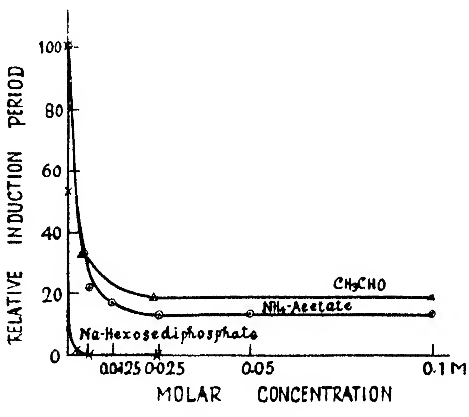

Fig. 4.

Thus it is concluded that among all the salts employed, NH_4 acetate produces the greatest effect in reducing the period of induction. When its effect is compared with those of Na hexosediphosphate and acetaldehyde, it will be seen (Fig. 4) that NH_4 acetate is a little more potent than acetaldehyde in reducing the period of induction, although Na hexosediphosphate is most effective.

Effect of salt mixtures.

In order to ascertain the combined effect of anions or cations it is necessary to keep the concentration of the accompanying ionic components constant.

Table V.

(A) Mixture of Na_2SO_4 and $(NH_4)_2SO_4$ in the presence of 0·04 M Na_2HPO_4.

Na_2SO_4 (M)	$(NH_4)_2SO_4$ (M)	Relative induction period	NH_4^+/Na^+
0	0·1	31	5
0·025	0·075	36	1·15
0·05	0·05	43	0·56
0·075	0·025	45	0·22
0·09	0·01	57	0·077
0·095	0·005	88	0·037
0·1	0	>250	0

(B) Mixture of K_2SO_4 and KNO_3 in the presence of 0·04 M Na_2HPO_4.

K_2SO_4 (0·5 M)	KNO_3 (M)	Relative induction period	$\frac{1}{2}$ SO_4''/NO_3'
0·1	0	37	∞
0·075	0·025	51	3
0·05	0·05	66	1
0·025	0·075	128	$\frac{1}{3}$
0	0·1	219	0

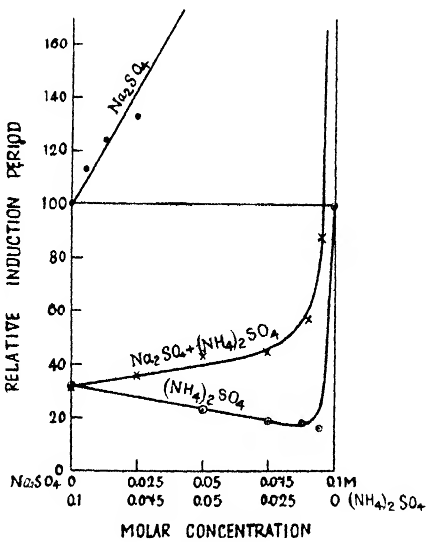

Fig. 5.

It will be seen from Table V (A), in which mixtures of Na_2SO_4 and $(NH_4)_2SO_4$ in various proportions are taken and the total concentration of sulphate is kept constant, that the period of induction is reduced as the concentration of NH_4^+ increases. When these values are compared with those of the individual effect of these salts, it will be seen (Fig. 5) that NH_4^+ is more potent than Na^+ in controlling the period of induction. In the presence of a small amount of NH_4^+ (0·01 M) the period of induction is remarkably reduced from the expected period (from about 200 to 57). The interpretation of these results is not very clear, but it is probably due to an antagonistic effect of NH_4^+ to Na^+.

When mixtures of K_2SO_4 and KNO_3, in which the total concentration of K^+ is kept constant, are employed, the sulphate ion exerts an antagonistic effect on the nitrate ion, although the effect is not so great as was found with NH_4^+ and Na^+ (Table V (B)).

In discussing the combined effect of Na^+ and NH_4^+, care should be taken to eliminate the effect of the sodium phosphate previously added. With this object, experiments were carried out with a mixture of NH_4 and Na phosphates in which the total concentration of phosphates was kept constant.

Table VI.

Mixture of Na_2HPO_4 and $(NH_4)_2HPO_4$.

(A) 0·04 M phosphate:

Na_2HPO_4 (M)	$(NH_4)_2HPO_4$ (M)	Relative induction period	NH_4^+/Na^+
0·04	0	100	0
0·038	0·002	33	0·05
0·036	0·004	21	0·11
0·032	0·008	18	0·25
0·02	0·02	11	1
0·008	0·032	9	4
0	0·04	4	∞

(B) 0·08 M phosphate:

Na_2HPO_4 (M)	$(NH_4)_2HPO_4$ (M)	Relative induction period	NH_4^+/Na^+
0·08	0	>300	0
0·06	0·02	33	$\frac{1}{3}$
0·04	0·04	28	1
0·02	0·06	21	3
0	0·08	18	∞

It will be seen in Table VI (A) and (B) that the period of induction is reduced in each case (0·04 M and 0·08 M phosphate) as the concentration of NH_4^+ increases.

When the relative induction periods are plotted against the ratio of NH_4^+ and Na^+ (Fig. 6), the curves are of much the same character. A hyperbolic relation can be supposed to exist between the relative induction period (= T) and the ratio of the two ions (= R); thus

$$T \times R^{\frac{1}{n}} = \text{constant},$$

where n is a constant according to the nature of the salt; with 0·04 M phosphate $n = 3$ and with 0·08 M phosphate $n = 4·86$.

If the ratio of NH_4^+ and Na^+ is based on the total concentration of Na ion (added Na_2SO_4 plus sodium phosphate previously added), it will be seen (Fig. 6) that the curve of 0·1 M sulphate + 0·04 M phosphate (which is incorporated in Table V (A)) is again of much the same character, and the above equation is also applicable when $n = 5$ is chosen as the constant for this mixture. It will be considered from these results that a simple antagonistic relation exists between the two cations NH_4^+ and Na^+; NH_4^+ acts in opposition to Na^+ in reducing the period of induction.

Fig. 6 shows that this kind of antagonism is also found between the two anions of sulphate and nitrate.

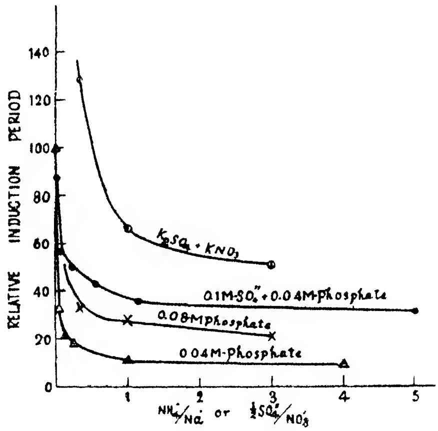

Fig. 6.

Summary.

1. Fermentation by dried yeast in the presence of 0·04 *M* phosphate was observed with various concentrations of inorganic and organic salts.

2. The period of induction was found to vary with the concentration and with the specific nature of the salt; both the cation and the anion.

3. The order of the potency of the salts in prolonging the period of induction was found to be $\frac{1}{2}$ $Ca^{++} > Na^+ > K^+ > \frac{1}{2}$ $Mg^{++} > NH_4^+$ with chlorides and sulphates, and $I' > Cl' > \frac{1}{2}$ SO_4'' with Na and K salts.

4. It was found that NH_4 salts (acetate, lactate, succinate and sulphate) were nearly as potent as acetaldehyde in reducing the period of induction, although they were less potent than hexosediphosphate.

5. The combined effect of the two ions was examined. NH_4^+ produced a remarkable antagonistic action to that of Na^+, and sulphate ion a slight antagonistic action to that of nitrate ion.

6. A hyperbolic relation may be supposed to exist between the induction period and the ratio of the concentrations of the two ions.

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LXXVI. BLOOD-FAT[1].

I. PREPARATION AND GENERAL CHARACTERISTICS.

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UNTIL adequate information regarding the chemical nature of the substances in which fatty acids are present in blood-fat is available, little progress can be made with the problem of the part which phosphatides, cholesteryl esters or other compounds may play in the transport of fat to the tissues. That such information is lacking is demonstrated by the following brief reference to the literature on blood-fat.

The earlier results record figures for the total fatty acids determined in many cases by use of the modification of the Kumagawa-Suto saponification method [1908] devised by Shimidzu [1910]. Latterly, an endeavour has been made, notably by Bloor [1915] and Bloor, Pelkan and Allen [1922] to obtain more detailed information by micro-analysis, in which estimations of total fatty acids, free and combined cholesterol and ether-soluble phosphorus have been carried out; and in this way evidence has been brought to bear on the question of the distribution of the fatty acids in the different combinations in which they may occur in blood. Consideration of the many results recorded shows, however, that very wide variations in any given value exist, and in some cases the findings are directly opposed. It is true that some of these variations may be accounted for by the fact that insufficient attention has been paid to the state of absorption of the animal whose blood has been investigated, but the impression remains that many of the results are due to the employment of methods ill-suited to the problem.

Although this abundance of quantitative data exists, there is a dearth of information concerning attempts to prepare substances in any state of purity from blood-fat. From time to time, claims to have obtained individual substances from blood have been made, such as those of Hürthle [1895] to have

[1] It will be shown during the discussion of the results that glycerides are virtually absent from the blood of the fasting ox and that almost all the material consists of phosphorus-containing compounds of fatty acids, together with cholesterol and its esters. Although "fat" is thus absent, we shall refer to the ether-soluble material throughout as "blood-fat" for the sake of ease of description, for the generic word "lipide" proposed by the International Congress of Applied Chemistry and used by Bloor [1925] has not found acceptance in this country, and the variety of terms used by other workers is confusing not only to the reader but to those engaged in the same field.

prepared the oleyl, palmityl and stearyl esters of cholesterol from serum, and of Bürger and Beumer [1913], who prepared sphingomyelin and kephalin from the stromata of red cells. Bloor [1923] prepared the fatty acids of plasma and corpuscles and studied their iodine values, and the same author [1924] prepared the fat from quantities of plasma up to 2 litres. To an ether solution of this fat he added excess of acetone, hydrolysed the precipitate and studied the iodine values of the fatty acids so obtained. The acetone-ether-soluble fraction was fractionally crystallised from alcohol and each fraction was then saponified and the iodine values of its constituent fatty acids determined. As a result of these latter researches, Bloor suggests that the unsaturated cholesteryl esters present are either the linoleate or a mixture of the oleate with the esters of acids more unsaturated than oleic acid. It will be remembered also that the ether-soluble phosphorus of blood, of which so many estimations have been made, is usually recorded as lecithin, although little information other than that just mentioned is available regarding its nature, obviously on account of the difficulty of working on an adequate scale. Our object, therefore, was to attempt to prepare blood-fat in a quantity sufficient to enable us to submit it to chemical examination with a view to obtaining more definite information as to the compounds in which phosphorus and cholesterol occurred and in the hope that such a study might result in our being able to express an opinion as to the validity of the results obtained by the many micro-methods of blood-fat estimation.

Another question which we have borne in mind is whether calcium phosphatidate, which may be regarded as the parent acid of lecithin and kephalin [Channon and Chibnall, 1927], is present in blood. Kephalin preparations usually contain calcium which can be removed by shaking with mineral acid [Diaconow, 1867; Thudichum, 1884; Parnas, 1909]. Calcium phosphatidate, like kephalin, is insoluble in alcohol and its calcium may be similarly removed, so that it is possible that this substance may be responsible for the calcium which usually contaminates kephalin preparations. If calcium phosphatidate is present in blood, the rise in ether-soluble phosphorus of the blood after a fatty meal, which has been observed and investigated by Bloor [1915], might be due to that substance which is a possible intermediate in the synthesis of lecithin and kephalin from the absorbed fat. Further, the study of the nature of blood-fat in a fasting animal may throw light on the hypothesis of Leathes and Raper [1925], who consider that the fatty acids of adipose tissue may be desaturated in the liver and that after being converted into highly unsaturated liver phosphatides they may be transported to the tissues in this form.

Before attempting to submit a large amount of blood-fat to fractionation into its various constituents, it was considered wise to prepare a number of samples and to make determinations of the fat constants and of phosphorus and nitrogen on the material in order to gain some idea as to its constancy of composition or otherwise, and to obtain some guidance for the treatment of the large preparation.

Preparation of blood-fat.

Our object in the preparation has been to obtain as much fat from blood with as little alteration of its original character, either by oxidation or by enzyme action, as possible. Blood contains rather less than 0·5 g. of fat per 100 cc., some of which is held by the protein in a form which prevents it from being easily removed. The difficulties encountered in attempting to remove this fatty material from so complex a tissue as blood are such that any method must necessarily be a compromise at many points, and one of them is well illustrated by the work of Shimidzu [1910], who found that the well-known saponification method of Kumagawa and Suto [1908] for the estimation of the total fatty acids in tissues cannot be applied to blood, a finding which we confirm. Shimidzu, therefore, devised a method in which blood is run into several volumes of alcohol and, after shaking, the mixture is allowed to stand for some hours. It is then filtered, and the protein residue is extracted for 6–8 hours by exposure to the vapours of boiling alcohol by suspension in a thimble over the surface of the solvent in a flask fitted with a reflux condenser. This alcohol extract is united with the original alcohol filtrate and the mixture is saponified and the fatty acids prepared by the Kumagawa and Suto method. In spite of this rigorous treatment, some material which on hydrolysis gives rise to fatty acids still remains in the protein residue, and these fatty acids, which can only be recovered by hydrolysis of this residue, amount to not more than 5 % of the total [Mayer and Schaeffer, 1913]. The material obtained by the alcohol extraction (prior to saponification) by the Shimidzu method is usually dark red in colour and contains a large proportion of substances which do not contain fatty acids but which are soluble in fat solvents, possibly because of the presence of the blood-phosphatides. Hence such an extraction method, although of great use for estimating the total fatty acids in blood, cannot be utilised for the preparation of blood-fat on account of the high proportion of extraneous material introduced, the length of time necessary for extraction with likelihood of consequent oxidation, and the impossibility of extracting large amounts of blood-protein in an atmosphere of hot alcohol vapour.

The alternative methods for obtaining the blood in a suitable form for extraction by fat solvents are precipitation by a solvent miscible with water or direct drying with suitable precautions against oxidation. No satisfactory method of direct drying being found, we were compelled to adopt a precipitation procedure in which we used alcohol.

Little advantage is gained by adding more than three volumes of alcohol to each volume of blood, for the increase in the percentage of alcohol in the filtrate by the addition of one or more volumes beyond three is relatively insignificant. It was decided to submit the protein residue to extraction with ether and not with alcohol as in the Shimidzu method, in order to avoid obtaining in the extract much material not of fatty nature to which reference

has been made. Such ether extraction will leave a definite amount of the fat unextracted, but a compromise at this point in favour of obtaining a less contaminated product which can be more rapidly prepared appeared desirable. The alcohol filtrate from the precipitation was evaporated to dryness and the dry residue also extracted with ether, and the extracts from the two fractions were combined. The relative amounts of material extracted from the protein residue and the alcoholic filtrate depended on the time during which the mixture of blood and alcohol was allowed to stand, but usually up to 80 % of the final material was obtained from the alcohol filtrate. Consideration was also given to the question as to whether it would be worth while to draw off the oxygen of the blood by exposure in a vacuum before precipitation in order to minimise oxidation (the solubility of oxygen in alcohol is about 20 times that in water). Accordingly experiments were carried out in which blood was treated by the method outlined above before and after removal of its oxygen, and iodine values were determined on the samples of fat obtained. Typical of these may be quoted the results of one experiment in which the iodine value of the fat of the blood directly precipitated was 58·2, and that of the same blood which had been previously sprayed into an evacuated flask was 58·9. In view of the fact that we were dealing with large quantities of blood, this difference was not considered sufficiently great to render it essential to remove the oxygen before treating the blood with alcohol. The material obtained by ether extraction of the protein residues was in all cases a waxy mass, golden yellow in colour, while that from the ether extract of the residue from the alcohol filtrates was reddish brown and contained much foreign material which was found to consist of nitrogenous substances. Since the bulk of the fat was obtained in the latter fraction, no useful purpose was served by keeping the two fractions apart and they were accordingly mixed.

Method.

Ox blood was received into oxalate at the slaughter-house and run into three volumes of alcohol within 30 minutes of being drawn. As the animals usually receive no food for 24 hours before slaughter, the fat of such blood may be regarded as at fasting level. The mixture was then vigorously shaken and allowed to stand for varying times, usually 0·5 to 2 hours. In the case of the 40 l. quantity, it was necessary to precipitate large volumes of blood at one time, and these stood in alcohol for varying times up to 24 hours. After standing, the mixture was filtered on a Büchner funnel. The residue so obtained needed considerable pressing before it became reasonably solid, and even then it was found to contain large amounts of solvent, for the volume of filtrate was never greater than 1·2 times the volume of alcohol added. The residue was then extracted in a Soxhlet apparatus until the ether extract was colourless. The original alcohol filtrate was evaporated to dryness in the water-bath at low pressure: after the volume had been rapidly reduced to about one-half, the solution frothed excessively. This frothing could only be

overcome by lowering the temperature of the water-bath at this stage and reducing the water pressure to the minimum necessary to maintain a vacuum, and evaporation was extremely slow. When the volume was reduced to about 10 % of the original extract frothing ceased and the evaporation could be continued as before. The last stages of the evaporation were also slow, for the fatty material which separated at the half-way stage then formed an emulsion, and only by raising the temperature of the water-bath to 80° could the water be effectively removed. In the final stages, absolute alcohol was repeatedly added to ensure efficient removal of the remaining water. Evaporation of the filtrate obtained from 1 litre of blood took 7-10 hours. The ether extract from the protein residue was also evaporated to dryness. The two residues were then washed out with ether into a stoppered cylinder and allowed to stand overnight. The solutions were filtered into weighed vessels, the solvent was removed, and a weight obtained after drying on the water-bath *in vacuo*. The material so obtained is usually reddish brown in appearance and, when warm, transparent; when cold it has the appearance (apart from colour) of a mixture of phosphatide and cholesterol, and is a sticky translucent mass. It contains, as we shall see later, a considerable amount of materials which are not truly ether-soluble. Its solution in ether is slightly fluorescent, and is usually turbid.

Determination of constants.

Aliquot portions of the ether solutions were used for duplicate determinations as set out in Table I. Nitrogen was determined by the micro-Kjeldahl method on 20-25 mg. quantities. The low phosphorus content (1–2 %) of the material would entail the employment of quantities of the order of 0·3 g. for a gravimetric estimation as magnesium pyrophosphate and we have been compelled in the routine determination of constants, and in the subsequent fractionation of the large batch of material on which a large number of phosphorus determinations has been necessary, to use the quinol-sulphite colorimetric method after oxidation of 8–12 mg. portions with sulphuric acid and "perhydrol." Cholesterol was determined by the Windaus [1910] digitonin method. The saponification values were determined on 0·5 to 1·5 g. portions by heating with 0·25 N (approx.) alcoholic potassium hydroxide for 4 hours. After titration, a considerable part of the alcohol was removed, and after being made strongly alkaline with potassium hydroxide the solution was poured into water in a separating funnel and exhaustively extracted with ether. The ether extract, after thorough washing with water, was evaporated to dryness and resaponified for one hour with sodium ethylate in alcoholic solution to ensure the complete hydrolysis of cholesteryl esters. After dilution with water, the mixture was again ether-extracted. The ether extract was thoroughly washed with water, and evaporated to dryness in a weighed flask. In this way the unsaponifiable fraction was obtained. It was, in all cases, a hard crystalline mass, of deep orange colour, the colour being

due to the lipochrome pigments carotene and xanthophyll: on this material total cholesterol was determined. The soap solutions from both saponifications, together with the washing of the ether solutions, were united and acidified with hydrochloric acid, and then extracted with ether. Difficulty has been encountered at this point, because acidification always causes the precipitation of a certain amount of slimy material which is insoluble in ether, and forms an emulsion at the ether-water interface. It has been our practice to leave this material suspended in the funnel with the first ether extract, and then to add to this ether extract the four or five successive ether extracts of the aqueous acid phase. The combined extracts were washed with water, until free from mineral acid, and then filtered. The insoluble material to which reference has just been made renders this filtration tedious. As to its nature we have no information, but such emulsions are encountered in the Shimidzu method of estimating blood-fat as pointed out by Mayer and Schaeffer [1913] and seem unavoidable with this type of material. The ether solutions of the fatty acids were evaporated, and dried *in vacuo* on the water-bath. The residues were dissolved in warm light petroleum (B.P. 40–60°) and allowed to stand overnight for the so-called "resinous" materials to separate out. After further filtration and washing of the residues, the light petroleum extracts containing the fatty acids were evaporated to dryness and weighed. Determinations of the iodine values (Wijs) and the molecular weight by titration were then carried out. The results are recorded in Table I.

Table I. *Analysis of the ether extract.*

(All figures are expressed as a percentage of the ether extract.)

Sample No. ...	1	2	3	4	5	6	7	8	9	10	11	12	Mean
Vol. of blood used, cc.	400	300	40,000	1200	1000	1000	350	350	350	350	500	400	—
Yield, g./l.	4·60	5·11	3·97	—	4·73	4·19	4·72	4·90	4·56	5·47	6·01	6·65	4·99
N	2·48	2·09	2·39	2·15	2·39	2·99	2·37	2·38	1·96	2·11	2·44	2·46	2·35
P	1·51	1·57	1·57	1·65	1·62	1·63	1·66	1·64	1·56	1·57	1·37	1·52	1·57
Unsap. matter	31·55	32·08	31·81	34·85	26·44	27·16	27·83	32·29	31·45	34·45	29·16	30·14	30·76
Free cholesterol	17·74	16·04	17·04	22·46	14·40	15·68	17·41	15·80	17·3	15·1	13·50	12·88	16·27
Cholesterol as ester	8·22	6·96	6·43	7·88	8·85	7·14	8·59	7·18	10·39	14·08	13·21	15·50	9·53
Unsap. matter other than cholesterol	5·59	9·08	8·34	4·51	3·19	4·34	1·83	9·31	3·76	5·27	2·45	1·76	4·95
Fatty acids	36·0	30·0	32·5	34·8	37·7	34·9	37·9	30·7	38·3	35·5	32·7	35·3	34·7
Iodine value	—	—	100	91·6	87·8	89·3	87·5	94·4	92·9	97·3	116·2	117·7	97·5
Mol. wt.	—	—	294	298	298	304	311	294	310	294	307	305	301

Discussion.

The proportion of fat extracted.

The figures in Table I show that the mean average phosphorus content of 12 samples of fat is 1·57 % P (extreme values 1·37, 1·66 % P), which corresponds to 7·78 mg. P per 100 cc. blood. Determinations of the ether-soluble phosphorus of 15 samples of ox blood gave a mean value of

9·8 mg./100 cc., which suggests that the method used has resulted in the extraction of 80 % of the ether-soluble phosphorus present. In order to gain further information as to the proportion of material obtained, the fat was prepared from a number of samples of blood, and at the same time determinations of the sum of the amounts of unsaponifiable matter and fatty acids were made on 50 cc. portions of blood by the Shimidzu [1910] method, save that the protein residues were not hydrolysed. In the calculations of the percentage yield allowance has been made for the fact that up to a further 5 % of material may be obtained from the hydrolysis of these residues. The results are recorded in Table II.

Table II. *Fatty acids and unsaponifiable matter extracted, calculated on the yields obtained by the Shimidzu method* [1910].

(Results expressed in mg./100 cc. blood.)

Fatty acids	179·1	150·4	174·9	194·4	212·0	239·0
Unsaponifiable matter	152·4	136·3	143·5	189·0	170·4	180·4
Total	331·5	287·6	318·4	383·4	382·4	419·4
Fatty acids and unsap. matter (Shimidzu)	369·6	361·4	365·2	433·8	430·0	515·6
Percentage yield	85·2	75·3	82·8	84·0	84·5	77·2

The mean figure for the yield expressed as a percentage of that of the Shimidzu method is 81·5 %. This figure is similar to that obtained for the proportion of phosphorus extracted, and it seems justifiable therefore to consider that the fat extract is probably fairly representative of the whole.

One further point needs mention. With small quantities of blood, ether extraction of the protein residue is readily accomplished. With larger quantities, such extraction is more difficult, and although every care was taken to see that thorough extraction was obtained, it is possible that some of the variations in yield may be accounted for to some extent by this fact. This remark applies in particular to the 40 l. quantity.

We have discussed the method of preparation and the question of the proportion of fat extracted somewhat fully because it was the one finally adopted, and because that used by Bloor [1924] in the simpler problem of preparing fat from plasma seemed to us unsatisfactory.

Consideration of Bloor's results, in which 16 samples of ox blood-plasma varying in volume from 500 to 2880 cc. were used, shows that the yield of phosphatide fatty acids varies from 0·0021 to 0·0799 g./100 cc., while in 5 cases apparently no phosphatide fraction was obtained, for the yields of fatty acids are not recorded. These results may be calculated in terms of ether-soluble phosphorus (fatty acids = P × 18·26), and such calculation shows that the average yield of fatty acids from the phosphatide fractions corresponds to an extraction from the plasma of 1·06 mg. of ether-soluble phosphorus per 100 cc. (extreme values 0·11 and 4·37 mg.), whereas the average figure obtained by us in direct determination of the ether-soluble phosphorus of 12 samples of ox blood-plasma is 6·62 mg./100 cc. Hence the method used by Bloor appears to have resulted in his obtaining an average

yield of not more than 16 % of the phosphatide fatty acids present in the plasma. Bloor also points out that early in his work it was discovered that the fat obtained contained free fatty acids which were removed and weighed. In this connection, analysis of the results from the four samples for which the data are most complete shows that in these four cases the material contained from 10 to 33 % of its content of fatty acids as free fatty acid, and these free fatty acids must have resulted from decomposition of some part of the fat during the preparation. Bloor comments on the low yield of fatty acids from the phosphatide fractions, and suggests that "insoluble residues are formed at certain points in the procedure which probably contained the altered phospholipoid; also, that in carrying out the various processes some of the lipoid was probably decomposed. The fact that a considerable amount of free fatty acid was always present in the lipoidal material, which would hardly have existed in the plasma itself, bears out the latter explanation." A material such as this, containing a large proportion of decomposition products, and only a small proportion of the phosphatide present in the plasma, which phosphatide may or may not be representative of the whole in the degree of unsaturation of its acids, appears to us to be unsatisfactory, and the conclusion drawn from its analysis open to doubt.

The fat prepared from blood by our method has never contained free acid, while it has been pointed out that its phosphorus content corresponds to an extraction of about 80 % of the ether-soluble phosphorus present in the blood.

In view of Bloor's experience with plasma, we submitted 25 cc. portions of 11 samples of plasma to the method which we have used for whole blood, in order to determine what proportion of the ether-soluble phosphorus was extracted, and whether the material contained free acid. The results are recorded in Table III. In no case did the extracted fat contain free acid. It is seen that about 80 % of the ether-soluble phosphorus present in the plasma has been obtained in the fat.

Table III.

(Plasma: ether-soluble phosphorus determinations and percentage of phosphorus extracted.)

													Mean
Ether-soluble P, mg./100 cc.	6·04	7·62	9·57	7·60	6·39	8·43	6·53	5·59	4·21	5·79	6·73	4·92	6·62
Wt. of fat, g./100 cc.	0·512	0·504	0·721	0·621	0·512	0·770	—	0·452	0·326	0·489	0·555	0·433	0·536
P as % of fat	1·03	0·91	1·19	1·08	0·92	1·02	—	1·04	0·89	0·91	0·87	1·01	0·99
P in fat as mg./100 cc. plasma	5·27	4·56	8·62	6·73	4·71	7·88	—	4·76	2·91	4·43	4·84	4·37	5·37
Percentage of P extracted	87	60	90	89	74	93	—	85	69	75	72	89	80

Absence of glycerides.

The initial observation to be made from the figures of Table I is that the sum of the average values of the total unsaponifiable matter (30·76 %) and of the fatty acids (34·69 %) is only 65·45 % of the material. This will be

accounted for in part by the fact that lecithin on hydrolysis yields but 70·3 % of its weight of fatty acids, but the greater part of the difference is due to the presence of extraneous nitrogen in the material. Before discussing this matter further, attention may be drawn to the amount of fatty acid which percentages of phosphorus and cholesteryl ester require[1], and which are set out in Table IV. It will be seen that there is, in general, fair agreement between the percentage of fatty acids found and those calculated on the basis of the phosphorus being present as lecithin and the cholesteryl ester as stearate[2].

Table IV. *Fatty acids.*

(Figures are expressed as a percentage of the fat.)

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12
Phosphatide fatty acids (P × 18·26)	27·6	28·7	28·7	30·1	29·6	29·8	30·3	29·9	28·5	28·7	25·0	27·7
Fatty acids combined with cholesterol (cholesteryl ester × [illegible])	6·0	5·1	4·7	5·8	6·5	5·2	6·3	5·3	7·6	10·3	9·9	11·4
Total	33·6	33·8	33·4	35·9	36·1	36·0	36·6	35·2	36·1	39·0	34·9	39·1
Found	36·0	30·0	32·5	34·8	37·7	34·9	37·9	30·7	38·3	35·5	32·7	35·3

If such calculations are justified, it is clear that there can be little glyceride present, a conclusion which the later fractionation of the fat from 40 l. of blood showed to be correct. In Table V are recorded the percentages of lecithin, cholesterol, cholesteryl ester and unsaponifiable matter other than sterol in the fat, what little glyceride there is present being neglected, the object being to gain an approximate figure for the percentage of non-fatty material present.

Table V.

(All figures are expressed as a percentage of the fat.)

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12
Lecithin (P × 26)	39·22	40·78	40·78	42·85	42·07	42·34	43·11	42·59	40·52	40·78	35·82	39·48
Cholesterol	17·74	16·04	17·04	22·46	14·40	15·68	17·41	15·80	17·36	15·10	13·50	12·88
Cholesteryl stearate	13·89	11·76	10·86	13·30	14·95	12·06	14·51	12·13	17·54	23·77	22·32	25·00
Unsap. matter other than cholesterol	5·59	9·08	8·34	4·51	3·19	4·34	1·83	9·31	3·76	5·27	2·45	1·76
Total	76·44	77·66	77·02	83·12	74·61	74·42	76·86	79·83	79·18	84·92	74·09	79·12
Residue; not fat	23·56	22·34	22·98	16·88	25·39	25·58	23·14	20·17	20·82	15·08	25·91	20·88

[1] From this point onwards, calculations involving the phosphatide will be made as has been done by previous workers on the basis of stearyl-oleyl-lecithin (N = 1·74 %, P = 3·85 %, fatty acids = P × 18·26); the cholesteryl ester will be regarded as cholesteryl stearate, an assumption which will subsequently be shown to be sufficiently accurate for the purpose of calculation.

[2] As pointed out on p. 668, difficulty has been encountered in extracting the fatty acids from the acidified soap solution on account of the presence of emulsions. Another difficulty which we have failed to overcome is that on no occasion have we obtained fatty acids which appeared "clean," in spite of the fact that attempts were made to remove what appeared to be resinous materials by second and third treatments with light petroleum. The same difficulty has been encountered by Rosenthal and Trowbridge [1915] using the Shimidzu [1910] method. Hence we cannot feel confident that the figures recorded in the above table are more accurate than to within possibly 5 %. We are investigating this matter further.

Presence of excess of nitrogen.

The sum total of these constituents is never greater than 83 %, and up to 25 % of the material consists of nitrogenous substances, as is shown by the excess of nitrogen over what is required by the phosphatide present. In the following table, the nitrogen percentage in excess of what is required by the phosphorus content and a N : P ratio of unity is shown, together with the percentage of non-fatty material (from Table V); the nitrogen percentage of the latter has been calculated.

Table VI. *The excess nitrogen expressed as percentage of the fat.*

Sample No. ...	1	2	3	4	5	6	7	8	9	10	11	12
N required if P is calculated as lecithin	0·68	0·71	0·71	0·74	0·73	0·74	0·75	0·74	0·70	0 71	0·62	0·68
N found	2·48	2·09	2 39	2·15	2·39	2·99	2·37	2·38	1·96	2·11	2 44	2·46
Excess N	1·80	1·38	1·68	1·41	1·66	2 25	1·62	1·64	1·26	1·40	1·82	1·78
Residue not fat (from Table V)	23 56	22·34	22·98	16·88	25·39	25·58	23·14	20·17	20 82	15·08	25·91	20·88
N percentage in residue not fat	7·66	6·18	7·31	8·35	6·54	8·80	7·00	8 13	6·05	9·31	7·03	8·54

The fact that an alcohol extract of a tissue may yield phosphatide preparations which contain far more nitrogen than is required by the amount of phosphorus present was shown by MacLean [1912] in his investigations on horse kidney, where the alcohol extracts yielded phosphatide material having N : P ratios varying from 4 : 1 to 1·5 : 1. He found that this excess of nitrogen was due to the fact that in the presence of phosphatides certain unknown nitrogenous substances are carried into solution and act as though they possessed all the properties of the phosphatides themselves. They can be removed in one way only, namely, by precipitation of the phosphatide emulsion in water by the addition of acetone. The nature of the complex mixture of these nitrogenous materials which accompany the phosphatides is discussed at length by MacLean and Smedley-MacLean [1927] under the generic name carnithin, where it is shown that the average nitrogen content of various samples was 6 % nitrogen and that substances with a nitrogen content of 28·5 and 40 % have been isolated from the mixture. It may be wondered why no attempt was made to remove this material from the blood-fat. The reasons are that acetone precipitation of an aqueous emulsion of the material results, as would be expected, in considerable losses of material which remains in the aqueous phase. The process needs repetition, and further it was considered preferable to analyse the fat, contaminated as it was with this material, rather than to remove it and obtain a purer preparation which might not be representative of the whole. Attempts were made to obtain fat from blood which did not contain this excess nitrogen by the use of different solvents, but these were unsuccessful. That the nitrogen percentage of the non-fatty residue in our material is higher (7·58 %) than that recorded by MacLean (6 %) may be due to the fact that calculation has been made of the nitrogen

required by the phosphorus on the basis of a N : P ratio of 1 (lecithin and kephalin) whereas it will be shown subsequently that some part, at least, of the blood-phosphatide may be sphingomyelin (N : P = 2 : 1, fatty acids = P × 12), the presence of which in red cells Bürger and Beumer [1913] claim to have demonstrated. On the other hand, the high and variable contents of nitrogen in the different substances obtained from samples of carnithin show how complex a mixture it is, and hence a constant nitrogen content is not to be expected.

Fatty acids.

The average figures for the molecular weights and iodine values of the fatty acids are 301 and 97·5 respectively. Of the figures available concerning the iodine value of blood-fatty acids, mention may be made of the finding by Imrie [1915] of an iodine value of 73 in a case of lipaemia, and of the results of Boggs and Morris [1909], who found that the iodine value of the fatty acids of the blood of rabbits rendered lipaemic by bleeding varied from 105 to 134. Csonka [1918] obtained a value of 87 for the liquid fraction of the mixed fatty acids of normal human blood (48 % of the total fatty acids) which corresponds to a value of 41. Bloor [1923] found an average iodine value of 147 for the liquid fraction of the fatty acids from 16 samples of ox blood-plasma. The liquid acids constituted 74 % of the total acids which, therefore, had an iodine value of 109 as against 97·5 found by us for those of whole blood. The higher figures obtained by Bloor may be due to the fact that he removed from the acids during the lead salt separation about 15 % of material, the lead salt of which was insoluble in hot alcohol and which on decomposition yielded a residue of low iodine value. The weight of this material was not included in the calculation of the percentage of the total acids which the liquid fraction represented, and its inclusion would lower the iodine value of the mixed acids to about 100. There is little doubt that the iodine values of the blood-fatty acids are higher than those recorded in this paper, for the nature of the starting material and the lengthy operations involved in their preparation must result in some degree of oxidation occurring in spite of the precautions taken to avoid it. Further, as has been pointed out already (p. 671 note 2), we think that the fatty acids have been contaminated by some other material which would also tend to lower the iodine values. That such contamination may exist appears to be borne out by the fact that the average molecular weight of the fatty acids is 301 (stearic acid has M.W. 284). Although, as will be shown subsequently, we have obtained from blood what appears to be lignoceric acid (M.W. 368), the amount present does not seem to be sufficiently great to account for the high molecular weight of the blood-fatty acids.

We have discussed all results in terms of percentages of the fat itself, and for the more physiological considerations some of them have been converted into yields expressed as mg. per 100 cc. of blood in Table VII.

Table VII.

(All figures are expressed as mg./100 cc. blood.)

Sample No. ...	1	2	3	5	6	7	8	9	10	11	12
P	6·95	8·02	6·23	7·66	6·83	7·83	8·03	7·11	8·59	8·23	10·10
Fatty acids combined with P (P × 18·26)	126·9	146·4	113·8	139·9	124·7	143·0	146·7	129·9	156·8	150·3	184·5
Fatty acids combined with cholesterol	27·83	26·16	18·78	30·80	22·01	29·83	25·88	31·85	56·63	58·41	75·82
Total fatty acids	154·7	172·6	132·6	170·7	146·7	172·8	172·6	164·8	213·4	208·7	260·3
Total fatty acids found	165·6	153·5	129·0	178·3	146·2	178·8	150·4	174·7	194·2	196·4	235·0
Free cholesterol	81·62	81·96	67·64	68·13	65·69	82·15	77·43	78·89	82·6	81·14	85·66
Cholesterol as ester	37·82	35·56	25·53	41·86	29·91	40·54	35·18	47·37	77·00	79·39	103·0
Unsap. matter other than sterol	25·71	46·39	33·11	15·09	18·19	8·64	45·6	17·15	28·82	14·72	11·70

If allowance be made for the amount of material other than fat present in the ether extract, it may be calculated that the yield of fat varies from 3·05 to 5·34 g./l. with a mean value of 3·97 g./l. The yield of fatty acids varies from 1·29 to 2·35 g./l., giving an average figure of 1·73 g./l., while the corresponding figures for the unsaponifiable fraction are 1·138 and 2·004 g./l., and 1·520 g./l. respectively. Thus the sum of the yields of unsaponifiable matter and fatty acids is 3·267 g./l., and the unsaponifiable fraction thus constitutes 46·8 % of this material, with values varying not greatly from the mean, viz. 42·3 to 49·5. Bloor [1923] obtained for the sum of the unsaponifiable matter in fatty acids an average yield of 3·01 g./l. from plasma, and found that the unsaponifiable matter constituted 43 % of this total. The unsaponifiable matter contains on the average 84 % of sterol precipitable by digitonin.

Although the saponification values of the fats were determined, they have not been recorded because the presence of the nitrogenous material already discussed vitiates them. They were carried out in the hope that if any considerable amount of glyceride were present in any sample of fat it would make itself obvious by raising the saponification value obtained. The mean of these values was 117.

Summary.

1. A method is described by which 80 % of the fat present in blood may be extracted. The material so obtained contains considerable quantities of nitrogenous substances.

2. Fat has been prepared from 12 samples of ox blood at fasting level, and a study has been made of its composition by determination of the fat constants and of phosphorus and nitrogen.

3. The amount of fatty acid present varies little from that required if the phosphorus of the fat be calculated as lecithin, and the cholesteryl ester as cholesteryl stearate, which suggests that glycerides are not present in appreciable quantity.

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LXXVII. THE UNSAPONIFIABLE FRACTION OF LIVER OILS.

V. THE ABSORPTION OF LIQUID PARAFFIN FROM THE ALIMENTARY TRACT IN THE RAT AND THE PIG.

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The researches of a number of workers, which have been undertaken in recent years in an attempt to elucidate the question as to the chemical nature of the unsaponifiable fractions of liver oils, have revealed the fact that such fractions may contain a number of hydrocarbons and alcohols of varying chemical structure. Of the alcohols, mention may be made of the occurrence in certain fish-liver oils of batyl, selachyl and chimyl alcohols [Tsujimoto and Toyama, 1922; Toyama, 1923, 1; 1924, 1, 2; Drummond and Baker, 1929]. The chemical nature of these dihydric alcohols was studied by Tsujimoto and Toyama [1922] and by Toyama [1924, 1] and later by Heilbron and Owens [1928] and Drummond and Baker [1929], who showed that they were the monoglyceryl ethers of octadecyl, oleyl and cetyl alcohols respectively. The presence in fish-liver oils of cetyl and oleyl alcohols has also been reported [Toyama, 1922; 1923, 1], while the latter alcohol has been found also in arctic sperm oil [Tsujimoto, 1925]. In addition to these alcohols, the presence of three hydrocarbons in various liver oils has been demonstrated. The unsaturated hydrocarbon, squalene, occurs in a large number of shark-liver oils [Tsujimoto, 1906; 1917; 1920, 1, 2] and, in traces, in cod-liver oil [Drummond, Channon and Coward, 1925; Drummond and Baker, 1929], and has been shown by Heilbron and his colleagues [Heilbron, Kamm and Owens, 1926] to be a dihydrotriterpene. In mammalian liver, a hydrocarbon of similar chemical nature to squalene occurs [Channon and Marrian, 1926]. A third hydrocarbon is *iso*octadecane which has been obtained from fish-liver oils by Tsujimoto [1917] and Toyama [1923, 2].

The question thus arises as to the source and possible function of these alcohols and hydrocarbons, and in particular whether they are present in these unsaponifiable fractions as a result of their occurrence in the food

ingested, or whether they are synthesised by the organism and play some part in the metabolism of fats or substances of allied nature. With these possibilities in mind, we have been investigating the ability of the mammal to absorb these unsaponifiable substances from the alimentary tract. The study of the absorption of substances other than fats but resembling fats in physical properties began with the researches of Munk, who found that when he administered cetyl palmitate to a patient with a lymph fistula he was unable to demonstrate the presence either of the ester or of the alcohol in the chyle [Munk and Rosenstein, 1891]. Later Connstein [1899] reported that almost all of the 20 g. of lanolin which he administered to a dog was excreted in the faeces, while Henriques and Hansen [1900] on administering a mixture of vaselin and lard to rats found that 95 % of the vaselin was excreted. Again, Bloor [1913] administered a liquid hydrocarbon mixture and also vaselin alone and in emulsions in olive and cocoanut oils, and recovered in the faeces 85–100 % of the hydrocarbons administered. He also gave similar mixtures to animals with thoracic fistulae and prepared the unsaponifiable fraction from the chyle. This was found in every case to be a solid melting about 110° (cholesterol has M.P. 149·5°). These results, therefore, show that although these unsaponifiable substances can be readily emulsified in the small intestine, they remain unabsorbed; and, since fats themselves are readily absorbed, such results provide weighty evidence against the occurrence of particulate absorption. Some of the more recent work, however, seems to suggest that unsaponifiable substances may be absorbed to some extent, at least. Thus, Thomas and Flaschenträger [1923] reported that cetyl alcohol was absorbed to some extent by the dog, and that the rat can also absorb this substance has been shown by Carter and Malcolm [1927] and Channon and Collinson [1928]. In addition, the latter authors carried out experiments with oleyl alcohol and phytol, and absorption of both these alcohols was found to occur in rats. The results of the experiments with phytol, cetyl and oleyl alcohols suggested that their absorption had caused an increase in the amount of unsaponifiable fraction of the liver, although the difficulty of identifying such substances in the small amount of material available prevented their actual isolation. The fact that they are absorbed, however, would possibly explain their appearance in the unsaponifiable fractions of certain liver oils.

The presence of hydrocarbons in the unsaponifiable fractions of liver oils suggested that a study of the question whether hydrocarbons could be absorbed from the small intestine should be undertaken afresh, and it was shown in a previous paper [Channon, 1926] that the unsaturated hydrocarbon, squalene, was absorbed by the rat and appeared in the liver. This finding, coupled with the occurrence of *iso*octadecane in fish-liver oils, made it appear worth while to investigate the question as to whether liquid paraffin was absorbed from the alimentary tract. Our reasons for re-opening this question in the light of much contrary evidence have been that considerable difficulties arise in determining whether absorption of unsaponifiable substances

in small amounts occurs. Hitherto, evidence has been sought in one of two ways. Substances have been administered by mouth and the chyle has been collected and examined for the particular substance, or, alternatively, after administering a known amount of the substance an attempt has been made to determine the amount excreted in the faeces during the experimental period. Apart from the known experimental difficulties of the first method, there is the greater one that the identification of unsaponifiable substances generally is one necessitating a large amount of material and, although the unsaponifiable fraction of the chyle fat may be prepared, the separation of unsaponifiable substances of similar chemical and physical properties is impossible, unless an individual substance constitutes a considerable proportion of the material available. The intake and excretion method suffers from the same difficulty, and in most cases the difference between the weights of the unsaponifiable fraction of the faeces from the control group of animals and that from those receiving the substance has to be taken as the amount of the substance excreted; an uncertain proceeding when it is remembered that faeces normally contain a high and variable content of unsaponifiable substances.

Since the results with squalene showed that this hydrocarbon could be isolated from the animal's liver after absorption, and those with cetyl and oleyl alcohols and phytol also suggested that some part at least of any unsaponifiable material absorbed by an animal would be found in that organ, we decided that a study of the unsaponifiable fractions of the livers of animals which had received liquid paraffin in their diet might provide a more delicate test as to whether the latter were absorbed. As liquid paraffin is a mixture of saturated hydrocarbons, the iodine value of the unsaponifiable fraction of the livers of animals receiving it in their diet should show a fall compared with that of the livers of the animals receiving the control diet only. The unsaponifiable fraction of mammalian livers consists largely of cholesterol, which may constitute up to 85 % of the fraction. Hence it will be necessary to remove the cholesterol quantitatively by means of digitonin, and determine the iodine value of the sterol-free material so obtained, or alternatively to determine the iodine value of the unsaponifiable fraction and to estimate quantitatively the amount of sterol present in it. Calculation of the iodine value of the sterol-free material may then be made, by making allowance for the amount which the cholesterol present contributes to the iodine value. A difficulty with the latter method has hitherto been that cholesterol behaves abnormally with iodine solutions [Smedley-MacLean and Thomas, 1920]. This difficulty has now been overcome by the introduction of the pyridine sulphate dibromide method of Rosenmund and Kuhnhenn [1923] as described by Dam [1925]. This method has been shown by Dam and later by Copping [1928] to give theoretical values with cholesterol, and it possesses the further advantage that it can be applied to very small quantities.

Experimental.

Exps. 1 *and* 2. *Administration of liquid paraffin to rats.*

A control group of rats was fed on the synthetic diet described by Drummond and Coward [1920], while 5 % of liquid paraffin, without previous emulsification, was mixed with the diet of the experimental group. The animals were maintained on these diets for 5 weeks, after which they were kept without food for 24 hours and killed. Their livers were removed and weighed, and the unsaponifiable fractions prepared from them by the method previously described [Channon, 1925]. Aliquot portions of the unsaponifiable fractions were then used for determination of the sterol content by the digitonin method [Windaus, 1910] and of the iodine value [Dam, 1925]. In a second experiment the procedure was exactly repeated. The results so obtained are recorded in Table I.

Table I.

	Animals receiving control diet		Animals receiving control diet plus liquid paraffin	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
No. of animals	5	6	6	6
Wt. of livers, g.	32·0	39·7	41·0	43·5
(*a*) Wt. of unsap. fraction, g.	0·0965	0·1447	0·1860	0·2122
(*b*) Wt. of cholesterol in (*a*), g.	0·0692	0·1200	0·0810	0·0936
(*c*) Wt. of material other than sterol in (*a*) (by difference), g.	0·0273	0·0247	0·1050	0·1186
Iodine value of (*a*) (Dam)	78·6	77·7	47·2	45·1

The results are rendered more lucid by the figures in Table II, in which are recorded the yields of unsaponifiable material per 100 g. of liver and the percentage of cholesterol in the unsaponifiable fraction. With the knowledge of the cholesterol percentage and that cholesterol has an iodine value of 65·8, it is possible to calculate, from these figures and the iodine value of the unsaponifiable matter, the iodine value of that part of the latter which is not sterol. Thus in the control group of Exp. 1, the unsaponifiable matter, which has an iodine value of 78·56, contains 71·69 % of sterol. The iodine value (x) of the non-sterol fraction may thus be calculated as follows:

$$\left(\frac{71{\cdot}69}{100} \times 65{\cdot}8\right) + \frac{28{\cdot}31}{100}\, x = 78{\cdot}56.$$

The results so obtained are also recorded in Table II, together with the mean values of the figures from the two experiments.

From the mean values it is seen that the yield of unsaponifiable matter from the livers of the animals which received liquid paraffin has increased by 40 % over that of the control groups, which suggests that some of the liquid paraffin has been absorbed and has appeared in the liver. This result seems to receive support from the degree of unsaturation of the non-sterol portion of the unsaponifiable fractions, for, whereas the iodine value of the material other than sterol is 118·8 in the control group, it is only 30·8 in the

case of the animals to which liquid paraffin had been administered. Moreover, the percentage of non-sterol material in the unsaponifiable fraction of the livers of the control group is only 22·68, as against 56·17 in the case of the animals which received paraffin.

Table II.

Exp. No.	Animals receiving	(a) Unsap. matter per 100 g. liver g.	Cholesterol in (a) %	Material other than cholesterol in (a) %	Iodine value of (a)	Iodine value of the non-sterol fraction of (a) (calc.)
1	Control diet	0·3016	71·69	28·31	78·6	110·9
2	,,	0·3645	82·94	17·06	77·7	135·8
1	Control diet plus liquid paraffin	0·4536	43·56	56·44	47·2	32·7
2	,,	0·4877	44·10	55·90	45·1	28·8
Mean values	Control diet	0·3330	77·82	22·68	78·1	118·8
	Control diet plus liquid paraffin	0·4707	43·83	56·17	46·1	30·8

With the other results already reported [Channon, 1926; Channon and Collinson, 1928] figures for the amount of unsaponifiable matter per 100 g. liver and its percentage content of cholesterol have been obtained from determinations on upwards of 200 rats. For this large number of determinations the mean value of the unsaponifiable fraction is of the order of 0·42 g. per 100 g. liver, of which 73 % is sterol precipitable by digitonin, while 27 % is composed of material other than sterol. These figures are mentioned here because, although the percentage of sterol in the unsaponifiable fraction of the control groups in the experiment under discussion is of the same order, 77·82, the yield of unsaponifiable matter per 100 g. of liver, 0·3330 g., is considerably lower than the average value worked out on the large numbers of livers to which reference has just been made. As to the reasons why the yields of unsaponifiable matter per 100 g. of liver in the control groups of these two experiments are considerably below the mean value found for the large number of animals, we have no information, for the diet in this and previous experiments has been the same, although the ages of the rats have varied. Hence no reliance can be placed on conclusions drawn from the figures obtained in these experiments for the yield of unsaponifiable matter per 100 g. of liver only. On the other hand, the significant result of these experiments is that, in the control animals, the material other than sterol in the unsaponifiable fraction constitutes but 22·68 % of the whole and has an iodine value of 118·8 as against 56·17 % and 30·8 for the corresponding figures in the animals which received paraffin. These results seem fairly convincing proof that some liquid paraffin has been absorbed and has found its way into the liver, to cause an increase in the amount of the unsaponifiable fraction and a consequent reduction in the percentage of cholesterol present and the iodine value of the material other than cholesterol. On the other hand, as has been pointed out already, the iodine values of the material

other than sterol have been calculated from the iodine value of the whole unsaponifiable matter by making allowance for the amount of cholesterol present. Since cholesterol gives theoretical results by the iodine method employed [Copping, 1928], this calculation seems to be sound. On the other hand, sterols tend to behave abnormally towards iodine solutions and, since these results seem directly opposed to those of the workers discussed in the opening paragraph of this paper, we have carried out a further experiment on larger animals (pigs) and attempted to isolate the hydrocarbon fraction in some state of purity, to avoid the doubts which calculated results inevitably raise.

Exp. 3. Administration of liquid paraffin to pigs.

Two pigs received a diet consisting of a mixture of barley meal 12 parts, white fish meal 1 part, and sharps 6 parts. Before the morning feed, one of the animals received in addition 100 cc. of liquid paraffin administered in the following way. To 100 cc. of gum acacia mucilage (gum 4 parts, water 6 parts) were added 100 cc. of liquid paraffin, and the mixture after very vigorous shaking was thoroughly incorporated in a small portion of the dry diet and given before the morning meal; it was readily consumed by the animal. After 54 days, the animals were killed 36 hours after their last meal. The unsaponifiable fraction of their livers was then prepared and the amount of the sterol present in an aliquot portion estimated by the digitonin method. The results obtained are recorded in Table III.

Table III.

	Animal receiving control diet	Animal receiving control diet plus liquid paraffin
Wt. of liver, g.	1537	1730
,, unsaponifiable fraction, g.	5·6231	8·2420
,, cholesterol, g.	4·231	4·267
,, material other than cholesterol, g.	1 3921	3·975

Note. As to the normal yield of unsaponifiable material from pig liver, we have little information. Two samples bought from shops at different times were worked up for their unsaponifiable content; in one case 935 g. liver yielded 3·47 g., and in the other 659 g. yielded 2·40 g., *i.e.* 3·71 and 3·64 g. per kg. respectively as against the control figure in the present experiment of 3·66 g. per kg.

With a view to the later isolation of the hydrocarbon mixture, it was decided to remove the sterol quantitatively. Accordingly the greater part was obtained by crystallisation of the fractions from light petroleum, boiling below 60°, with which liquid paraffin is miscible. Each fraction was dissolved in 110 cc. of light petroleum and cooled to 0°. The crystalline material obtained by filtration was washed with light petroleum cooled to 0° and the combined mother-liquors were concentrated to 50 cc., and the process was repeated. In this way, there were obtained 3·35 g. and 3·40 g. of sterol from the fractions from the livers of the control animal and the animal which received the liquid paraffin respectively. The remaining sterol was removed

with digitonin. To each of the residues obtained by evaporation of the light petroleum mother-liquors were added 50 cc. of 95 % alcohol, and the mixtures heated to boiling and vigorously shaken. A considerable portion of the material from the liver of the animal which received paraffin failed to dissolve. To each there was then added 3·1 g. of digitonin dissolved in 240 cc. of boiling 90 % alcohol. After gently boiling the mixtures with vigorous shaking, they were allowed to stand overnight. They were then filtered through paper, and the residues, consisting of cholesterol digitonide, excess digitonin and any substances insoluble in 90 % alcohol, were washed with the latter solvent and the washings were united with the mother-liquor, which was then evaporated to small bulk and poured into water. The mixture was then ether-extracted, and the extract washed with water, evaporated to dryness and dried *in vacuo* on the water-bath. The residues on the filters were then thoroughly washed with ether and the ether extract was filtered into a separating funnel. This ether extract was also washed with water and evaporated to dryness, and dried on the water-bath. By this process, each fraction was freed from cholesterol, and divided into two portions, one soluble in about 350 cc. of 90 % alcohol and the other insoluble in that volume of this solvent. The object of the procedure, apart from removing cholesterol, was to concentrate any liquid paraffin present in the insoluble fraction, for the liquid paraffin used in the feeding of the animals was found to have a solubility in 90 % alcohol at room temperature of only 0·18 g./100 cc. Iodine values were then determined by Hübl's method on the four fractions obtained. The weights of the four sterol-free fractions and their iodine values are recorded in Table IV.

Table IV.

	Animal receiving control diet	Animal receiving control diet plus liquid paraffin
Sol. in 90 % alcohol, g.	0·9589	1·3182
Insol. in 90 % alcohol, g.	0·4969	2·5785
Iodine values:		
Sol. in 90 % alcohol	92·8	57·7
Insol. in 90 % alcohol	151·2	25·7

From the figures recorded in this table, the iodine values of the combined sterol-free fractions for each animal may be calculated. In the case of the control animal, the material other than sterol has an iodine value of 112·8 and for the animal which received the paraffin 36·6. These figures may be compared with those obtained in the experiments on rats, the mean values of which were 118·8 and 30·8 respectively, and confirm the results obtained on those animals by a less direct method.

Before describing the attempts made to isolate the hydrocarbons, it will be well to consider the results recorded in Table IV and to discuss the nature of the substances which present-day knowledge suggests may be present. If liquid paraffin be present, it would be expected that the greater

part of the fraction containing it would remain insoluble in the volume of 90 % alcohol used for precipitation of the cholesterol by digitonin. In addition, the fact that liquid paraffin has a slight solubility in 90 % alcohol should cause the amount of the soluble fraction in the case of the animal receiving paraffin to be greater than that in the control. The figures in the table record such a result, for the amount of the insoluble material in the animal receiving paraffin is about five times as great as in the control animal, while there is a corresponding increase of about 0·36 g. in the soluble fraction. Since the paraffin has a solubility of 0·18 %, it might have been thought that this latter difference would be greater considering the volume of solvent used. The reason for it not being so is probably that the separation was carried out in the presence of the flocculent precipitate of cholesterol digitonide which carried down the paraffin with it. It was in the hope of this happening that the procedure adopted was used. A consideration of the iodine values should tell us something of the nature of the substances present. If the soluble material in the case of the animal receiving paraffin contains some hydrocarbon, it should have a proportionately low iodine value. This is so, for the control material has an iodine value of 92·8 as compared with 57·7 for that of the animal receiving paraffin. The differences shown in the insoluble fractions are still more considerable, for the control material has an iodine value of 151·2 as opposed to one of 25·7 for the animal receiving paraffin. It was decided, therefore, to attempt to isolate the saturated hydrocarbons from the fractions insoluble in 90 % alcohol.

Fractions insoluble in 90 % alcohol. It was shown in a previous paper [Channon and Marrian, 1926] that there was present in mammalian liver a highly unsaturated hydrocarbon, similar to squalene. This hydrocarbon was not obtained in pure form, but its presence was deduced from the fact that it was possible to isolate it as a bromide and hydrochloride. Since it is very insoluble in any solvent containing water, it should appear in this experiment in the fractions insoluble in 90 % alcohol, and the iodine values of those fractions will be due mainly to the presence of this unsaturated hydrocarbon. It was decided to remove it by taking advantage of the fact that the bromide to which it gives rise is insoluble in ether. Accordingly, the fractions were dissolved in anhydrous ether at 0° and excess of a solution of bromine in ether was added. After standing for a short time, the insoluble bromide from the two fractions was removed by filtration and washed with ether. Each was shown to be the bromide of the unsaturated hydrocarbon, to which reference has just been made, by its bromine content (68 %) and by the fact that it charred at 180° without melting. In this way there were isolated 0·37 g. and 0·5 g. of insoluble bromide from the material from the liver of the control animal and the animal which received paraffin respectively. Since the fraction from the liver of the latter animal had an iodine value of 25·75, 2·45 g. should absorb 0·3975 g. of bromine, while the bromine content of the insoluble bromide isolated is 0·34 g. Hence the bromine content of the

material from which the unsaturated hydrocarbon has been removed is 0·0575 g., or 2·45 % bromine, and hence, prior to bromination, the material present other than the unsaturated hydrocarbon had an iodine value of 4 only. The filtrate and washing from the insoluble bromide were then shaken with sodium thiosulphate solution in order to remove the excess of bromine; and the ether solutions, after washing with water and drying with anhydrous sodium sulphate, were evaporated to dryness. In view of the low bromine content (2·45 %) it was decided to distil the material without debromination. The 2·2 g. were transferred with ether to an 8 cc. flask but, unfortunately, about 20 % of the material was lost during the removal of the ether by evaporation. The material was distilled at 1 mm. pressure. There were obtained two fractions and a residue: fraction (1), 0·3 g. boiling up to 160°, fraction (2), 0·9 g. boiling from 160°–210° and 0·32 g. of residue. The distillates were amber-coloured oils of viscosity similar to that of liquid paraffin.

Fraction (2) analysed as follows:

0·1021 g. gave 0·3202 g. CO_2 and 0·1262 g. H_2O: C, 85·53; H, 13·73.

0·1482 g. absorbed 0·31 cc. of 0·1036 N iodine: Iodine value 2·7.

These figures show that fraction (2) approximates sufficiently closely to a saturated hydrocarbon as to render it unnecessary to attempt to purify the remainder further, and the object of this experiment, namely the isolation of the paraffin from the liver, was thus achieved.

Fractions soluble in 90 % alcohol. It was considered desirable to attempt to demonstrate whether the increase in the amount of the fraction soluble in 90 % alcohol derived from the liver of the animal which received the liquid paraffin was caused by the presence of that substance. Accordingly, these fractions, 0·864 g. and 1·186 g. from the control and the animal receiving paraffin respectively, were converted into acetyl derivatives by boiling with acetic anhydride. The acetyl derivatives were then saponified by boiling with excess of 0·2 N alcoholic potassium hydroxide for 4 hours and the acetyl values so determined. After saponification, the fractions were recovered by ether extraction and brominated in cold dry ether as described in the previous section. After removal of the insoluble bromide of the small amount of the unsaturated hydrocarbon appearing in these fractions, the excess bromine of the mother-liquor was removed by washing with sodium thiosulphate solution. The ether solutions were then dried with anhydrous sodium sulphate and evaporated to dryness, and the bromine content of the materials so obtained determined. The results obtained are given in Table V.

Table V.

	Animal receiving control diet	Animal receiving control diet plus liquid paraffin
Acetyl value	116·6	91·3
Wt. of insol. bromide (g.)	0·14	0·05
Acetyl value corrected for the unsat. hydrocarbon	123·5	92·5
Bromine content after removing the insol. bromide, %	25·68	22·14

It will be seen from the above table that both the acetyl values and the bromine contents of the fractions after bromination are considerably lower in the case of the fraction from the animal which received paraffin, and it seems clear, therefore, that the increased amount of the latter over that of the control animal is due to liquid paraffin appearing in it, which would lower its acetyl value and the percentage of bromine in the brominated product.

Discussion.

The results obtained from the experiment on the pigs in which hydrocarbon has been isolated in nearly pure form leaves no doubt that some part of the paraffin administered has been absorbed and has appeared in the liver. Further, the figures obtained showing the effect of the administration of liquid paraffin on the yields of the unsaponifiable fractions and their iodine values are similar in both the rat experiments and those with the pigs, and if due allowance be made for the biological nature of the experiment, these figures may be used to gain some insight into the question as to how much paraffin has accumulated in the liver of the animals. These figures are set out in Table VI, where the mean values of the two rat experiments are given.

Table VI.

Animals	Diet	Sterol g./100 g.	Non-sterol g./100 g.	Iodine value of non-sterol
Rats	Control diet	0·2591	0·0755	118·8
(mean values)	Control diet plus liquid paraffin	0·2063	0·2643	30·8
Pigs	Control diet	0·2752	0·0905	112·8
	Control diet plus liquid paraffin	0·2467	0·2298	36·6

In the rat experiments, the non-sterol fraction of the livers of the animals which received paraffin is roughly 3·5 times greater than that of those of the control animals, and the iodine value has shown a fall corresponding closely with this increase. In the pig experiment, the increase in the non-sterol fraction is about 2·5 times that of the control, while the iodine value is about one-third as great as the control iodine value, a result in not quite such good accordance as the rat experiments provide.

As to the mechanism by which the absorption of the paraffin has occurred, we have little information. In a previous paper [Channon and Collinson, 1928], the absorption of cholesterol, phytol, cetyl and oleyl alcohols was discussed. The results of Moore and Parker [1901], which extended the earlier observations that bile salt solutions would dissolve fatty acids, by demonstrating that such acids were very much more soluble in bile salt solutions in which lecithin was present, made it worth while to determine the solubilities of these alcohols together with that of squalene and liquid paraffin in bile salt solutions with and without lecithin. Not too great a reliance can be placed on these results on account of the difficulties of such determination and the very low solubilities obtained, but it was found that the alcohols were soluble to an extent in rough proportion to the degree to which they

were absorbed. The solubility of squalene in 5 % bile salt solution at 37° was found to be as low as 0·024 %, while liquid paraffin was found to possess no detectable solubility in this medium. In this connection, the results of Mellanby [1927, 1] are of interest. Mellanby introduced various fat emulsions into the duodenum of a cat and observed fat absorption by inspection of the lacteals in the mesentery, cannulae having been previously inserted into the gall bladder, common bile and pancreatic ducts. He found that emulsified fat to which bile had been added was rapidly absorbed, and absorption caused a secretion of pancreatic juice and an increased flow of bile. On the other hand, neither emulsified fat nor fat previously digested by lipase was absorbed in the absence of bile, and the injections of such mixtures caused no secretion of pancreatic juice and no increase in bile secretion. If there be no available source of lipase other than pancreatic juice, therefore, emulsified fat according to these results may be absorbed without previous hydrolysis. Continuing these experiments, Mellanby [1927, 2] found that in a similar experiment in which an emulsion of paraffin and bile was injected, there was neither absorption of the hydrocarbon nor secretion of pancreatic juice nor an increase in the bile secretion. An experiment which the same author kindly carried out with squalene showed that in contrast to liquid paraffin squalene was absorbed to a small extent, and that the injection caused a large secretion of pancreatic juice and bile. Mellanby's results, both with emulsified fat and squalene, suggest that particulate absorption may occur in spite of much contrary evidence. The results with liquid paraffin recorded in this paper are opposed to those just cited, and in this connection the work of Clark and Clark [1917] may be mentioned. These authors studied the results of the injection of fine emulsions of fat, such as olive oil, oleic acid, egg-yolk and cream, into the tissue of tadpoles' tails. They found that the fat injected was soon surrounded by leucocytes, and lymph vessels grew out to the oil droplet in a time varying from a few hours to two or three days. No reaction, however, was obtained with substances such as mineral oil.

A word may be said here as to the chemical nature of the liquid paraffin used in the experiments. The main fraction boiled at 210° at 1 mm., and since no solid products could be obtained by distillation, and by attempts at crystallisation of the undistilled residues, we were led to the view that the hydrocarbons present in the mixture could not be normal aliphatic substances. This view was confirmed by the figures obtained on combustion, for the mean values showed that the liquid paraffin contained C, 86·6 %; H, 13·3 %. On the other hand, $C_{10}H_{22}$ requires C, 84·50; H, 15·50 and $C_{20}H_{42}$ requires C, 85·1; H, 14·9, and hence a mixture of aliphatic hydrocarbons C_nH_{2n+2} of which liquid paraffin might be composed would have figures intermediate between these such as C, 84·8; H, 15·2, a carbon content definitely lower than that actually found. We consulted Dr W. R. Ormandy on this point and he informed us that, although the popular idea is that medicinal paraffin is composed entirely of aliphatic compounds, strong grounds exist for believing that a large

proportion of it is naphthenes and that some authorities go so far as to consider that the whole of ordinary liquid paraffin is composed of ring compounds. A naphthene $(CH_2)_n$ requires C, 85·7; H, 14·3, while our liquid paraffin corresponds to $(CH_{1\cdot 84})_n$. From the combustion figures, therefore, the liquid paraffin appears to consist of a mixture of polycyclic paraffins. This point is important because the nature of the hydrocarbons present in the paraffin may be a factor involved in the absorption[1].

We have no information as to how much liquid paraffin was absorbed by the animals in the experiments described in this paper, but the results of previous workers suggest that it can have been absorbed in small amount only. The fact that some absorption occurs, however, suggests that a study should be made as to whether liquid paraffin administered to a lactating animal can be detected in the milk. This is a question of some practical importance to which we are now giving attention.

Summary.

1. The iodine value of the non-sterol fraction of the unsaponifiable matter of the livers of rats which receive liquid paraffin in their diet was found to be 31 as against 119 for that from the control animals. This result suggests that liquid paraffin is absorbed to some extent.

2. This was confirmed by an experiment on pigs, in which the hydrocarbon was isolated from the liver.

3. The factors concerned in the absorption are discussed.

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[1] The question as to whether unsaturated material was present in the paraffin was investigated by determination of the iodine value (Wijs). The average value found was 0·3.

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LXXVIII. A SECOND THERMOLABILE WATER-SOLUBLE ACCESSORY FACTOR NECESSARY FOR THE NUTRITION OF THE RAT.

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During the last two years numerous papers have been published confirming the work of Goldberger *et al.* [1926] on the division of the water-soluble vitamin B into at least two components. That further factors may be present has been suggested by Kennedy and Palmer [1928], Hunt [1928] and others. The experiments to be recorded below deal exclusively with the more labile factors concerned. The work was undertaken in order to find the daily requirements of a rat of the antineuritic vitamin in terms of the pigeon day-dose, the method employed in this laboratory for standardising this vitamin. A preliminary account of the work has already appeared [Reader, 1928].

The nomenclature used throughout is that provisionally adopted by the Biochemical Society and defined in detail by Chick and Roscoe [1928]. Briefly, B_1 signifies the antineuritic, and more heat-labile factor; B_2, the more heat-stable factor.

Although control animals on our basal diet + crude marmite grew to adult size at the same rate as those on normal food (corn, bread, lettuce, etc.), repeated attempts to get growth to adult size on basal diet + marmite which has been subjected to alkaline hydrolysis for one hour at 120° + purified extracts of vitamin B_1 have failed. Thus it would seem that at least two factors necessary for rat nutrition had been destroyed by this treatment. Whether or not this second thermolabile substance is identical with that described by Williams and Waterman [1928], as associated with the maintenance of weight and general conditions of adult pigeons, will be discussed later.

It is proposed to call this second thermolabile rat factor, provisionally, vitamin B_3.

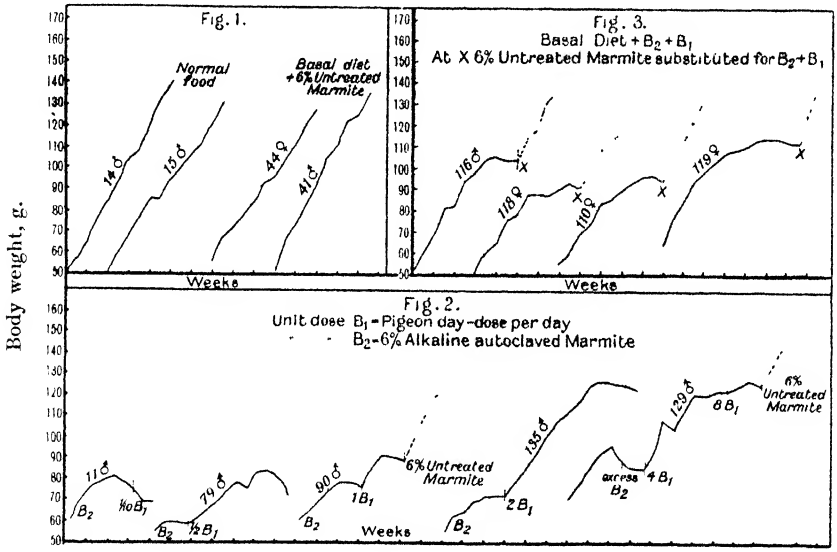
Experimental.

Albino rats, bred in this department from the Wistar strain supplied by Glaxo Limited, were used throughout. In all routine experiments animals of 50–60 g. were used for these tests. However, in order to compare these experiments with those reported by the Lister Institute, one set of animals of about 40 g. was put on to the experimental diet (see Fig. 5).

The basal diet used was:

Glaxo caseinogen (free from vitamins A, D, B_1 and B_2) ...	20 %
Rice starch	70
Agar-agar	2
McCollum's salt mixture	5
Cod-liver oil	3
Water *ad lib.*	

This diet was freshly prepared daily, and was tested to show that, when supplemented with 6 % marmite as source of B_1 and B_2, it gave growth equal to that obtained with normal food, including corn, "Hovis" bread, lettuce, cod-liver oil, and occasionally milk. The curves in Fig. 1 are selected from groups of 20 animals and are typical of the group. The daily food intake gradually rose from 10 to 15 g. as the body-weight increased from 60 to 120 g., so that in all each animal was having daily B_1 and B_2 equivalent to 0·6–1·0 g. marmite.



In all further experiments the thermostable factor or factors (B_2) were supplied by adding 6 % autoclaved marmite solution (120° for one hour at p_H 9·0) to the basal diet. Curative tests on pigeons showed that this product did not now contain any B_1, the antineuritic factor. That it did contain one factor necessary for the rat (presumably B_2) was shown by the method of Chick and Roscoe [1928] for the assay of B_2. Also that 6 % was sufficient for the daily requirements was demonstrated by doubling this quantity after growth ceased in one group of rats (*e.g.* rat 129, Fig. 2) and noting that no further growth occurred.

Two types of experiments were designed. In (A) the rats were fed on the basal diet + B_2 till constant weight was obtained for three consecutive

weighings (three-day intervals). Then varying doses of several B_1 concentrates were administered daily, in aqueous solution adjusted to p_H 6·5. The doses were given by the mouth from a small pipette. The concentrates used were prepared by Mr H. W. Kinnersley[1]. Unless otherwise stated they were the 50 % alcohol extracts from the charcoal. Daily doses were varied from 1/10 to 8 pigeon day-doses. Typical results from a large number of experiments are shown in Fig. 2. From these it may be seen that even eight pigeon day-doses per day do not give growth at a normal rate to adult size.

In (B) (Fig. 3) there was no preliminary lag period to constant weight as the animals were fed from the start on basal diet + B_1 + B_2. It will be seen from Fig. 3 that in every case growth ceased or proceeded at a very subnormal rate after 4 or 5 weeks. Immediately untreated marmite was substituted as source of B_1 and B_2 growth was resumed to maximum weight.

Thus it would seem clear from Figs. 1, 2 and 3 that a second factor, other than B_1, had been destroyed by the alkaline hydrolysis of the marmite.

0·1 N *HCl extracts of B_1.*

Experiments of type (A) above were arranged with B_1 concentrates prepared by extraction with 0·1 *N* HCl from charcoal in place of the 50 % alcohol extracts used above. Results were similar to those in Fig. 2, but slightly more marked, so presumably these extracts were more free from the second thermolabile factor than the 50 % alcohol extracts (see Fig. 4).

Use of younger animals.

As four weeks' feeding was needed in many of the above experiments before the absence of the second thermolabile substance became evident, it was now decided to carry out a set of experiments using younger animals (40 g. instead of 60 g.), in order to compare these results with those reported from the Lister Institute. As was expected, these animals were more sensitive to the lack of B_3; in fact, some of them died within a week of the time they began to drop in weight. However, if carefully watched and fed with untreated marmite they completely recovered (Fig. 5).

Properties of second thermolabile substance.

(*a*) It is generally agreed that alkaline hydrolysis for one hour at p_H 9 at 120° destroys the polyneuritis-curative properties of an aqueous suspension of marmite, yeast, etc. If this heating is carried out at a more acid reaction (p_H 6)[2] destruction of vitamin B_1 does not occur, but from the

[1] *Note by H. W. Kinnersley.* The concentrates used in the above experiments were the 0·1 *N* HCl or 50 % alcohol extracts from charcoal. In each case, after removal of metallic ions by H_2S, they were fractionated into 99 % alcohol [see Kinnersley and Peters, 1928, pp. 425, 426].

[2] I am indebted to Prof. R. A. Peters for testing these solutions. He has found that they retain at least 50 % of their curative properties when autoclaved under these conditions. The matter is still under investigation.

curves in Fig. 6 (rats 167 and 104) it would appear that there has been more than 50 % destruction of B_3. As would be expected, upon the view that B_1 is not destroyed, the early part of the curves is similar to those obtained with B_2 + the less impure extracts of B_1. Additional indirect proof was obtained by adding B_1 at the point where growth first ceased and noting that no increase in weight was observed (see Fig. 6, rat 104).

(*b*) From the above experiments it seemed clear that B_3 is more thermolabile than B_1. Further proof of this was obtained by boiling marmite in an open vessel on a water-bath for 2 hours (the actual temperature of the marmite was 95°–97°). More than 50 % destruction occurred. 12 % marmite in the diet did not give growth equal to that previously obtained with 6 % (Fig. 6, rat 152).

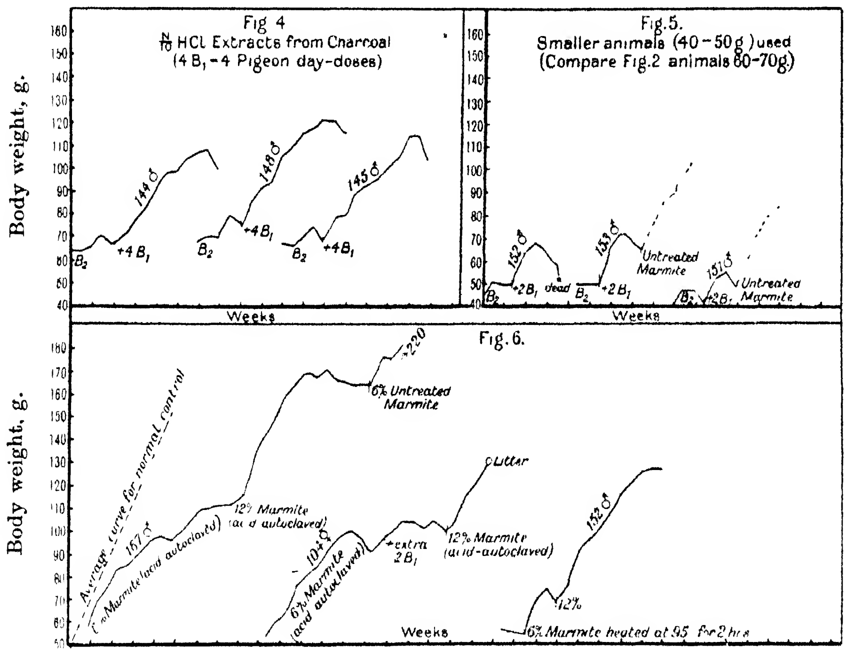


Fig. 6. Partial destruction of second thermolabile factor
(*a*) by acid hydrolysis at 120° for 1 hour;
(*b*) by heating at 95° for 2 hours.

(*c*) *Solubility in ether.* Six hours' extraction with ether in a Hurtley extractor removes most of the B_3. However, if the ether is now distilled off, the substance cannot be redissolved in fresh ether. Thus it does not seem to be a true solubility of the substance itself, but rather dependent on adsorption upon associated substances. Further work upon this point is being carried out in the hope of getting a supply of B_3 free from B_1.

Discussion.

From the above experiments it would seem established that when marmite is subjected to alkaline hydrolysis at 120° for one hour at least two factors necessary for rat nutrition are inactivated. One of these is the antineuritic vitamin B_1. It is suggested that the other is a second thermolabile factor B_3. Evidently B_1 concentrates can be prepared free from this second factor by the extended method of Kinnersley and Peters [1928], and it is due to the advantage of having these more pure concentrates at my disposal that I have been able to demonstrate the absence of the second thermolabile factor from diets complete in other respects. However, so far a sample of B_3 has not been prepared free from B_1: therefore it cannot be definitely proved that the lack of growth is not due to the racemisation of some amino-acid or similar substance. In fact this view is supported by the fact that acid hydrolysis at 95° and at 120° apparently cause a certain definite loss of efficiency (> 50 %) whether the heating be for one hour or two: but absolute destruction of both B_1 and B_3 occurs on alkaline hydrolysis at 120° for one hour.

Williams and Waterman [1928] have recently reported the presence of a very thermolabile factor in yeast necessary for the maintenance of the weight and general condition of pigeons. However, they were unable to show its necessity for the rat. Whether they observed complete destruction of their factor by heating is not stated. As their preparation of B_1, made by the fuller's earth method, was not heated in the process, the B_3 reported in my experiments was probably still present associated with their B_1; hence they would not get a cessation of growth due to lack of this factor. Moreover, certain preliminary experiments carried out in this department (Peters, private communication), indicate that the second rat factor does not restore the weight of pigeons; thus the evidence seems to be in favour of two further factors—one, reported by Williams and Waterman, for pigeons: the other, reported in this paper, for rats.

At first sight these results seem to be contradictory to the results of Chick and Roscoe [1928]. In their work on the assay of B_2, they used B_1 concentrates prepared by the method of Kinnersley and Peters [1927] but not purified by alcohol fractionation, so it is possible that traces of B_3 were still present. However, it must be emphasised that in their actually recorded experiments they assessed their results from the growth obtained in the first 2 weeks after addition of B_2 to diets apparently complete in other respects. This procedure employs the justifiable assumption that if a diet is deficient in more than one factor, an immediate response is obtained by addition of any one of these. If, however, they had continued their experiments for 4 or 5 weeks, the picture presented might have been a different one.

SUMMARY.

Evidence is presented for the destruction of at least two factors, necessary for the nutrition of the rat, by alkaline hydrolysis of yeast extract at p_H 9 at 120° for one hour.

The conclusion is therefore drawn that a second thermolabile factor (B_3) is present in yeast extract.

My thanks are due to Professor R. A. Peters for his interest and criticism throughout the work, to Mr Kinnersley for the supply of B_1 concentrates, and to the Medical Research Council for a grant.

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LXXIX. SOME EVIDENCE OF THE EXISTENCE OF A FURTHER FACTOR NECESSARY FOR GROWTH OF THE RAT.

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THE experiments described in this paper were designed to ascertain how far the vitamin A-free diet in use in this laboratory could, when supplemented by cod-liver oil, support normal growth. In the early work of this department (1927) there had been nothing in the behaviour of the rats used in assaying cod-liver oil to suggest any deficiency in the diet other than vitamin A, for the addition of this substance in a dose of 0·01 g. daily caused resumption of rapid growth in the test rats for a period of 9 weeks, after which the test was discontinued (Fig. 1). It was several months later that a

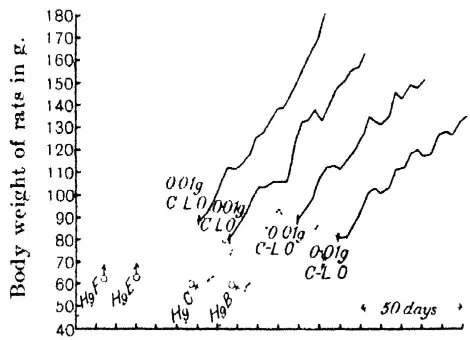

Fig. 1. A test for vitamin A in cod-liver oil. Rats given "vitamin-free casein" (Glaxo) throughout the experiment. Evidence that some rats, at least, can grow well when this casein is the source of protein.

deficiency other than vitamin A was suspected in the vitamin A-free diet and possibly also, though to a smaller extent, in the diet of the stock colony.

The history of the attempt to breed a colony of rats on some diet that should be uniform throughout the year may form the subject of a separate communication. The result of the first stage of it may be summarised as a progressive deterioration of the litters obtained from the imported mature animals, both in respect of their rate of growth when allowed to grow to maturity for breeding and later in their failure to rear the young they had borne. A progressive decrease in the periods of time required for the young

rats to become steady in weight when given the vitamin A-free diet, and a lower weight at which they ceased growth was also noticeable. The whole of these results could, so far, be attributed to a low vitamin A content in the diet of the stock rats with the consequent low reserves of this factor in the rats used for vitamin A assays. It became evident, however, that the responses to doses of cod-liver oil given by these particular rats were definitely less than the responses obtained in rats of different history. Moreover, increasing the dose of vitamin A gradually to a very high dose (0·2 g. daily) of a sample of cod-liver oil (which in other rats caused resumption of good growth in a dose of 0·01 g.) failed to bring about any increased rate of growth. It was evident that the diet was lacking in some factor other than vitamin A.

Coward, in 1926, discussed with Prof. Palmer his work on the growth-promoting powers of different samples of caseinogen prepared in different ways, and it appeared at once possible that the rats in the experiments described in this paper were suffering from a deficiency such as Palmer had noted in his work. The first substance examined for the unknown factor was therefore a fresh kind of caseinogen. The British Drug Houses "light-white casein" was substituted for the Glaxo "vitamin-free casein" of the diet previously used. The response was immediate and dramatic (Fig. 2). The rats grew rapidly and gave no further evidence of any deficiency in their diet. Although Palmer's work has finally indicated that his rats were suffering from a shortage of the vitamin B_2 [Palmer and Kennedy, 1927, 1, 2; 1928, 1, 2], yet further work in this laboratory has shown that the "light-white casein" (B.D.H.) contains neither B_1 nor B_2, but some growth-promoting property which is independent of both of these. The same factor has been found in various natural foodstuffs. It is at least partially destroyed by heat; it is extracted slightly from the casein by hot alcohol and by hot ether after cold alcohol. It seems to be more easily extracted by hot ether from wheat embryo. It is not vitamin E.

TECHNIQUE.

Rats from various colonies were used in the early tests carried out in this department. That they varied greatly in their reserves of vitamin A was evident from their varied behaviour when given the vitamin A-free diet of this laboratory. Litters were kept intact and members of one litter were, in general, exhausted of their reserves of vitamin A within a very few days of each other. There were much greater differences in vitamin A reserves between different litters than between different members of any one litter.

The vitamin A-free diet consisted of:

Dextrinised rice starch	71 %
"Vitamin-free casein" (Glaxo)	15
Agar-agar	2
Dried yeast	8
Salt mixture (Steenbock's)	4

The mixture was irradiated in very thin layers (200 g. over an area of 4 sq. ft.) in zinc trays for 30 minutes at a distance of 2 ft. from a quartz mercury vapour lamp, running at 2·5 amp., 130 v. It was stirred and re-spread after the first 15 minutes of irradiation. This method has been shown by Steenbock and Coward [1927] to generate ample supplies of vitamin D; and that it is also adequate with the materials available in this laboratory has been shown from time to time by experiment. Rats which had ceased growth on this diet have been given large daily doses of irradiated ergosterol and have not resumed growth.

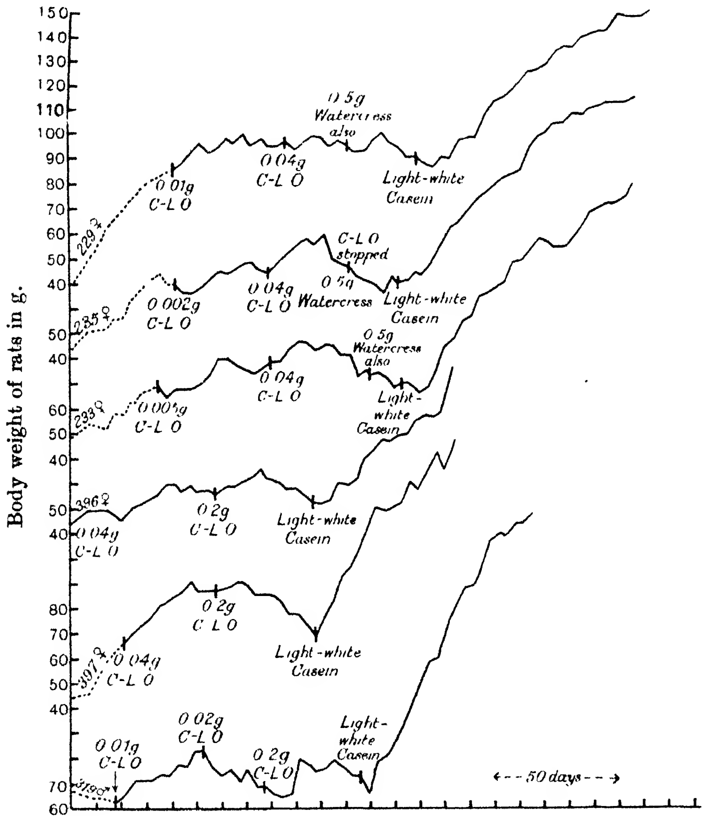

Fig. 2. A test for vitamin A. Rats given "vitamin-free casein" (Glaxo) at first. Growth ceased in spite of the doses of cod-liver oil being greatly increased and in spite of the addition of watercress. Growth was only resumed when "light-white casein" (B.D.H.) was substituted for the "vitamin-free casein."

The rice starch was partially dextrinised by mixing it with enough water to make it firm, and then heating it in shallow trays in an ordinary gas cooking stove for 6 hours, which renders it crisp and easily ground. The dextrinisation of rice starch does not seem to be necessary for making it more easily digested, as with the corn starch used in American laboratories. Dextrinisation is necessary, however, to get the starch into a less fine state of division, for in

its original state the rats collect a great deal of it in their fur and the whole experiment is less clean than when dextrinised and ground starch is used.

The "vitamin-free casein" supplied by the Glaxo Company was guaranteed substantially free from vitamin A by tests which were until recently supervised by the Accessory Food Factors Committee of the Medical Research Council and Lister Institute.

Agar-agar was incorporated in the diet as a preventive of constipation, although, in view of the large percentage of dried yeast also used, it is very doubtful whether it was necessary. Sometimes the powdered form of agar-agar was used, sometimes the shredded. When the latter was used it was eaten as readily as the rest of the food. It was not washed or otherwise purified before use.

The dried yeast used in this diet was brewer's yeast. Every sample purchased for use in this laboratory is tested for its vitamin B complex and there is ample evidence that the inclusion of 8 % of it in the vitamin A-free diet is abundant for the vitamin B needs of the rat at all stages of growth. The evidence rests on the ability of rats, from some colonies at least, to grow rapidly (bucks to more than 200 g., does to about 180 g.) when this is the only source of the vitamin B complex in their diet.

The rats were kept on screens to prevent coprophagy only when they were being used for a vitamin B test. Otherwise they were kept on shavings or sawdust.

The evidence for the existence of a growth-promoting factor other than the vitamins A, B_1 and B_2, D and E in "light-white casein" (B.D.H.).

The general scheme of the experiments which led to the detection of the absence of this factor from the "vitamin-free casein" of the vitamin A-free diet and of its presence in the particular sample of untreated (*i.e.* "light-white," B.D.H.) casein examined later, was as follows. In addition to those experiments with rats which had failed to give the expected response to doses of cod-liver oil, others were performed with rats which had given a certain amount of response under the same condition. In the latter experiments the vitamin A tests of the samples of cod-liver oil were carried on for much longer periods than that of 5–6 weeks usually considered necessary for determining the potency of the oil. In general, the rats ceased to grow and would not resume growth on being given larger and larger doses of cod-liver oil, or if growth were resumed it was only for a short time. Watercress, as an additional and different source of vitamin A, was then tried on some of these rats. A dose of 0·5 g. of it caused no resumption of growth, although an independent test [Coward and Eggleton, 1928] had shown that in the normal way of testing, when the rats were only about 10 weeks old, a dose of 0·1 g. had been enough to restore growth. Unlimited supplies of watercress were then given to the rats which had failed to grow on 0·5 g. of it, and although growth was resumed for a time, it was only temporary. Then "light-white casein"

(B.D.H.) was substituted for the "vitamin-free casein" (Glaxo) and rapid and prolonged growth was resumed. At this stage of the investigation, a discussion on vitamin A assay in cod-liver oil with Dr J. W. Trevan revealed the fact that he had encountered the same difficulty in raising rats to maturity on a vitamin A-free diet plus cod-liver oil. He had found that only by giving such rats large quantities of fresh grass clippings could they be made to resume growth.

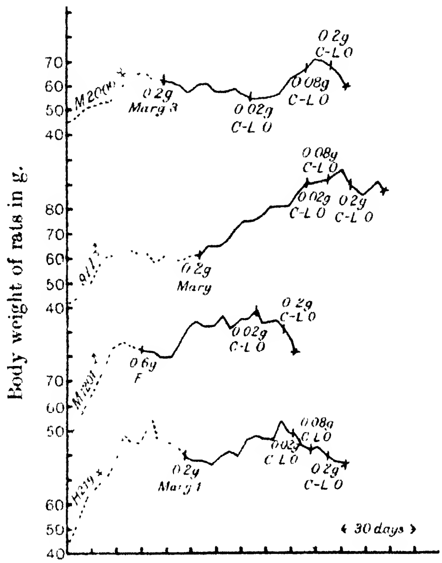

Fig. 3. Growth curves for comparison with those in following figures. If the lately recognised factor necessary for growth is withheld, death ensues. These are typical of some 70 such curves obtained during this work.

Other sources of the supplementary growth factor.

It has been for many months now a matter of routine in this laboratory to keep rats which have already been used for a vitamin A test for further work on the unknown factor. If they have not reached maturity at the end of the recognised test period, they are deprived of their vitamin A-containing substance until they begin to fall in weight. They are then given 0·02 g. daily of a good sample of cod-liver oil. If they do not resume growth within 2–3 weeks the dose is doubled and in time increased to 0·2 g. The great increase in dosage of cod-liver oil has seldom resulted in any increased growth.

It has therefore been possible to test several other food substances and preparations on a reasonable number of rats, for only the animals which had received very large doses of vitamin A from the beginning of the test period, and so had grown rapidly, or rats which had been used for assaying the vitamin A content of milk, failed to respond to the later treatment adopted for exhausting their reserves of the unknown factor.

The distribution of the factor, so far studied, may be summarised thus:

Table I.

Substance	Dose tested	No. of rats used	Results
1. "Light-white casein" (B.D.H.) untreated, 4 different samples	15 % of diet	40	Rapid growth in all but 3 rats, slow growth in these
	10 % of diet	4	Good growth but less rapid than when 15 % was given
	5 % of diet	4	No growth
2. "Crude casein" (B.D.H.)	15 % of diet	4	1 died. 3 maintained weight for 75, 49, 30 days respectively*
3. Dairy butter	0·5 g. daily	3	Maintained weight for 34 days
4. Fresh milk (London supply)	10 cc. daily	4	Rapid growth in all
5. Milk boiled for 15 mins.	10 cc. daily (= 15 cc. fresh)	4	Moderate growth in all
6. Lettuce—fresh	1 g. daily	7	Maintained weight in 5 for 7–30 days. Fair growth in 2
	Ad lib., often 40–50 g. per rat per day	7, the same animals that had 1 g.	Good growth
7. Etiolated wheat shoots, 10 days old (grown under laboratory conditions)	15 g. daily	3	Slight growth
8. Dried grass clippings	5 % of diet	3	Rapid growth in 2 rats. Slow growth in 1 rat increased by raising amount to 10 %
9. Fresh grass clippings	About 10 g. daily	2	Rapid growth
10. Extra dried yeast	10 % of diet	14	No influence*
11. Marmite	0·5 g. daily	13	Slight temporary increase in weight in 5. Maintained weight in others†
12. Autoclaved marmite	1·0 g. daily	3	No influence†
13. Watercress	*Ad. lib.*, about 10 g. daily	3	Slight increase†
14. Ox muscle	1 g. daily	4	Good growth
	Ad. lib, 15–20 g. daily	4	Very rapid growth
Ox liver	1 g. daily	4	Very rapid growth
15. Wheat embryo	5 %	3	Rapid growth in 2. Weight maintained in 1
	10 %	3	Rapid growth
16. Wheat germ oil, sample 0	Up to 0·08 g.	6	No influence‡
	Up to 0·16 g.	4	1 maintained weight. 2 fair growth. 1 died
Wheat germ oil, sample 1	Up to 0·02 g.	3	No influence
	Up to 0·12 g.	3	No influence in 2. Fair growth in 1
Wheat germ oil, sample 2	0·12 g. daily	4	Fair growth in 3. Maintained weight in 1

* One of these rats was finally given "light-white casein" to which it responded with rapid growth.

† Two of these rats were finally given "light-white casein" to which they responded with rapid growth.

‡ One of these rats was finally given 10 % wheat germ to which it responded with rapid growth.

Similar tests were made with (*a*) liquid paraffin (0·06 g. daily for 21 days) and (*b*) 3 % extra agar-agar in the diet to see whether the active substances had merely been exerting an influence on peristalsis. Each substance was without effect on the growth of the test animals.

The palatability of the two diets containing the "Glaxo casein" and the "light-white casein" respectively.

A simple experiment was carried out to see whether possibly greater palatability of the "light-white casein" diet could account for the observed results. Six young rats weighing about 60 g. were divided into two groups of 3 each. One group was given the vitamin A-free diet containing the "vitamin-free casein" (Glaxo) and the other the diet containing "light-white casein." All the rats were given 0·02 g. of a good sample of cod-liver oil daily to make the same rate of growth possible in all of them. Records of food consumed each day were taken at 9 o'clock in the morning in order to get figures as accurate as possible. After 2 days the diets of the two groups were interchanged. This was repeated 5 times in all and then each group was kept to one diet for a period of 5 days, and the diets were again interchanged for a further period of 5 days. The amounts consumed in g. are given in Table II.

Table II.

Rat	M 347		348		349		350		351		352	
Casein in diet	G.	L.-W.	G.	L.-W.	G.	L.-W.	G.	L.-W.	G.	L.-W.	G.	L.-W.
27. iii. 28	7	—	5	—	7	—	—	7	—	3	—	3
28	6	—	7	—	5	—	—	7	—	5	—	5
29	—	7	—	5	—	7	9	—	7	—	8	—
30	—	7		5	—	7	9	—	7	—	8	—
31 (2 days)	13	—	14	—	17	—	—	19	—	16	—	17
2. iv. 28	—	5	—	2	—	8	10	—	8	—	9	—
3	—	5	—	2		7	10		8	—	8	—
4	5	—	6	—	9	—	—	9	—	9	—	8
5	6	—	6	—	9	—	—	9	—	8	—	9
6	—	5	—	7	—	8	11	—	9	—	11	—
7 (2 days)	—	10	—	10	—	12	14	—	14	—	14	—
9	—	2	—	4	—	(Spoilt) 12?	4	—	4	—	4	—
10 (2 days)	—	15	—	20	—	20	20	—	21	—	19	—
12	6	—	9	—	8	—	—	10		9	—	9
13	7	—	9	—	11	—	—	12	—	10	—	10
14 (2 days)	12	—	17	—	22	—	—	28	—	22	—	27
16	8	—	10	—	11	—	—	12	—	10	—	11
17	—	8	—	10	—	14	14	—	12	—	11	—
Total for 11 days	70	64	83	65	99	95	101	113	90	92	92	99

An examination of this table shows that

(*a*) there is no significant difference between the total amounts of the two diets eaten by any one rat during the experiment, and that

(*b*) there are practically no instances of a greater consumption of the "light-white casein" diet on the first day of receiving it than of the diet containing the "Glaxo casein." The evidence shows rather the reverse of this.

Hence the possibility of these results being due to the greater palatability of the "light-white casein" diet must be discarded.

Effect of heat on the growth-promoting power of "light-white casein."

I. "Light-white casein" was heated in very thin layers in open trays in an electric oven (300 g. over 8 sq. ft.) for 24 hours at 105°. It was raked over twice during this time. It was then given to rats (5) as 15 % of their diet. They grew as well as the rats receiving 15 % of untreated "light-white casein" and it was evident that there had been no appreciable diminution in the potency of the casein.

II. "Light-white casein" was heated as above for 7 days, and then tested in the same way. Again the response was good but definitely less than before.

III. Milk which had been simmered for 15 minutes in an open pan lost a great part, though not all, of its activity.

Attempts to extract the growth-promoting substance.

I. *From "light-white casein."*

Extracts of the "light-white casein" made with (*a*) cold 97 % alcohol (4 times), (*b*) cold ether (4 times) after cold alcohol (once) and evaporation *in vacuo* both failed to cause any resumption of growth, while the casein so extracted showed no impairment of its growth-promoting powers. Similar results were obtained with a 2 % acetic acid extract, neutralised with potassium hydroxide and evaporated on a water-bath, and with the casein thus extracted.

Rather more active extracts were obtained by the use of (*a*) hot alcohol (4 extractions of 6 hours each) and of (*b*) hot ether after drying overnight (4 extractions of 6 hours each). The residual casein showed somewhat impaired activity. The extracts described above were all tested in doses equivalent to the amount of casein eaten per rat per day. Slight activity was also shown by an ether extract of a solution of casein in sodium carbonate, which was tested in a dose equivalent to 5 times the daily intake of casein. The growth-promoting factor is therefore not easily removed from the casein by the usual fat solvents.

II. *From wheat embryo.*

(*a*) *Hot* 90 % *alcohol.* Extracting wheat embryo with 90 % alcohol 4 times by boiling for 6 hours each time removes the active factor almost completely and gives an active extract. The yield of this extract was about 30 % (95 g. and 87 g. respectively from two portions of 300 g. each). When given to two rats in doses of 0·05 g. daily (equivalent to 0·15 g. wheat embryo), there was no response. When given in larger doses (1 g. and later 2 g.), one rat resumed rapid growth immediately and another after a period of about 30 days. Another, having remained steady on about 1 g. per day for 80 days,

resumed rapid growth when the amount was doubled. Three rats given the extracted wheat embryo grew very slowly for about 60 days but resumed rapid growth immediately non-extracted wheat embryo was substituted for the extracted product (Fig. 4).

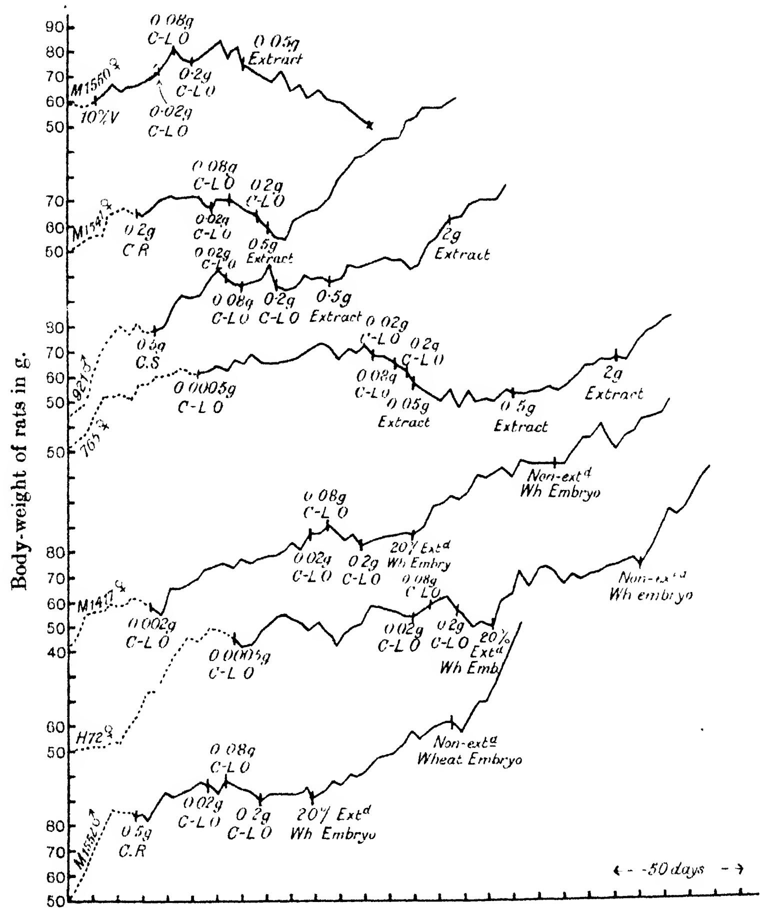

Fig. 4. A 90 % alcohol extract of wheat embryo. Definite activity of the extract and impairment of the activity of the wheat embryo.

(*b*) *Hot ether.* Wheat embryo was dried overnight in an oven at 105° and extracted 4 times with boiling ether. The ether was distilled off at atmospheric pressure. The extract was decidedly active in a dose of 0·16 g. and more so in one of 0·24 g. The extracted wheat embryo was definitely less active than the non-extracted product (Fig. 5).

Evidence from vitamin B testing of the existence of a growth-promoting factor in "light-white casein" (B.D.H.).

In view of the slight temporary response to extra doses of vitamin B in the form of marmite, which was occasionally experienced in the experiments

already described, "light-white casein" (B.D.H.) was tested first for the whole B complex, then for B_1 and B_2 independently. It was found to contain neither of these but to contain a third factor essential for growth and independent of B_1 and B_2.

Technique. The diet on which rats have been prepared for vitamin B testing in this laboratory (until a few months ago) was composed of:

Dextrinised rice starch	79 %
"Vitamin-free casein" (Glaxo)	15
Agar-agar	2
Salt mixture (Steenbock's)	4

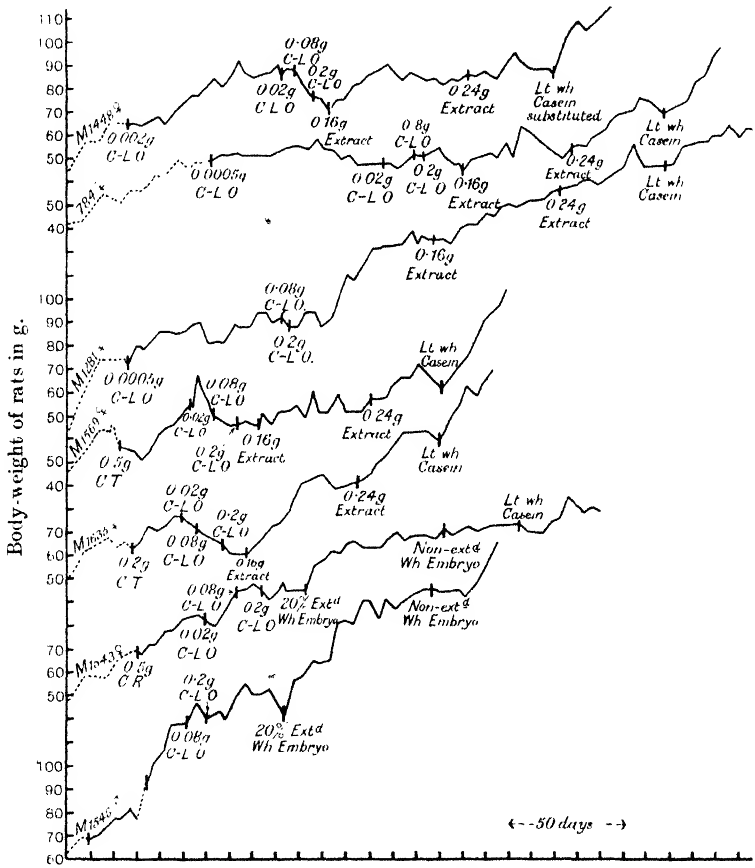

Fig. 5. A hot ether extract of wheat embryo. Some activity of the extract and slight impairment of the activity of the wheat embryo.

Each rat was given separately 5–6 drops of a good sample of cod-liver oil twice a week. In 10–14 days on this diet, the rats became steady in weight or declined.

To test "light-white casein" for the full vitamin B complex, it was substituted for the "Glaxo casein" (15 %) in the diet of 5 rats prepared as just

described. None of the animals showed any tendency to resume growth. They all declined in weight steadily and died in 10–20 days. The "light-white casein" was therefore considered to be devoid of the vitamin B complex.

To test "light-white casein" for B_1 and B_2 independently of each other, 8 rats were prepared on the diet described above. The scheme of the experiment was to determine whether "light-white casein" could produce growth if the rats were given in addition (*a*) excess of B_1, (*b*) excess of B_2, (*c*) excess of both B_1 and B_2.

The B_1 preparation used in this experiment was very kindly given to the authors by Prof. J. C. Drummond, who had found that the dose required to make a rat grow in the presence of ample supplies of B_2 was about four times the amount required to cure a pigeon of polyneuritis. The pigeon dose of this particular preparation was 0·1 cc. (confirmed in our own laboratories) and in order to ensure excessive doses of B_1, 0·8 cc. was used for each rat.

The B_2 preparation was also supplied by Prof. Drummond. It was made by autoclaving marmite (decidedly alkaline) at 125° at 15 lbs. pressure for 3 hours. It was then made very slightly acid with HCl and the volume made up so that 1 cc. was equivalent to 0·4 g. of the original marmite.

The 8 rats were divided into four groups and given the following doses:

Group I. 0·8 cc. B_1 preparation per rat per day.
1·0 cc. B_2 ,, ,,

Group II. 0·8 cc. B_1 preparation per rat per day.
"Light-white casein" in place of "vitamin-free casein."

Group III. 1·0 cc. B_2 preparation.
"Light-white casein" in place of "vitamin-free casein."

Group IV. 0·8 cc. B_1 preparation.
1·0 cc. B_2 ,,
"Light-white casein" in place of "vitamin-free casein."

After 16 days, the dose of B_2 was doubled, as several workers have declared lately that the vitamin B potency of marmite is somewhat less than formerly.

The result is seen in Fig. 6. Even the high doses of B_1 and B_2 have failed to produce normal growth in the animals of Group I. The animals of Group II have grown considerably less than those of Group I, thus showing that "light-white casein" supplies no appreciable quantity of B_2. The animals of Group III died sooner than did those of Group II, thus showing that "light-white casein" does not contain B_1. The animals of Group IV resumed rapid and prolonged growth till the experiment was terminated at the end of 8 weeks. It is therefore evident that "light-white casein" contains some factor necessary for growth which is not one of the vitamins A, B_1, B_2 or D.

Two questions then presented themselves.

(*a*) Why was any growth obtained either by ourselves or by other workers when rats, prepared as these, were given preparations of B_1 and B_2?

(*b*) Is the factor here demonstrated the same one as was demonstrated by the experiments previously described in this paper?

The first question can apparently be answered (*a*) by assuming the rat's ability to store an unknown factor such as is contained in the "light-white casein" or (*b*) by assuming the presence of this growth-promoting factor in the vitamin A-free diet, but in a much smaller degree than in the "light-white casein."

The second question can at present only be answered by repeating the tests on the substances used in the first set of experiments (vitamin A technique), using now the technique described immediately above; *i.e.* testing each substance by the vitamin B technique in the presence of excess doses of B_1 and B_2.

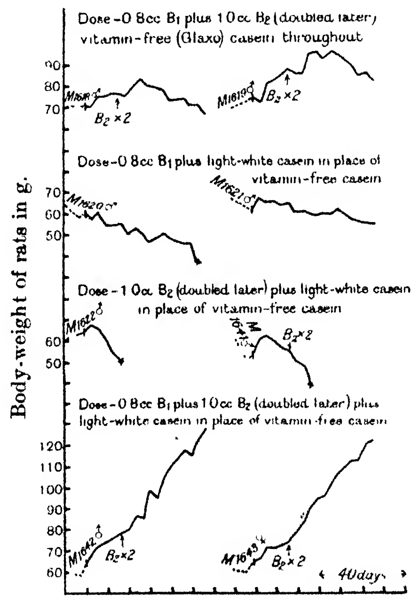

Fig. 6. To demonstrate the presence of a growth-promoting factor in "light-white casein" which is not in the "vitamin-free casein" and which is neither vitamin B_1 nor B_2.

Two experiments on this latter point have been carried out.

In the first experiment, 4 rats were prepared on the "B" diet containing the "vitamin-free casein" (Glaxo). After 11 days, they were all given daily doses of 2 cc. of the same preparation of B_2 as in the last experiment and 0·1 cc. of a B_1 preparation very kindly given to the authors by Dr H. Chick, who had found this dose of her preparation ample for the growth needs of the rat. One of the 4 rats continued to receive the "Glaxo casein"; it grew slowly and 21 days after the beginning of the test period had gained 17 g. in weight. Two rats were given "crude casein" (B.D.H.) in place of the "vitamin-free casein" (Glaxo) and at the end of the experiment (21 days) had gained 2 and 7 g. respectively. The fourth rat was given "light-white

casein" (B.D.H.) and had gained at the end of the experiment (21 days) 52 g. in weight. This would appear to indicate that the "crude casein" (B.D.H.) was as nearly free from the unknown factor as the "Glaxo casein."

In the second experiment a comparison was made between (*a*) the "vitamin-free casein" (Glaxo), (*b*) the "light-white casein" extracted once with alcohol and four times with ether in the cold, and (*c*) untreated "light-white casein." The same doses of B_1 and B_2 as in the last experiment were given to all the rats. At the end of 31 days, the one rat given "vitamin-free casein" had gained 17 g. in weight, the two given the extracted "light-white casein" had gained 37 and 32 g. respectively, while the one given untreated "light-white casein" had gained 49 g. It was evident that some of the active factor, but by no means all of it, had been extracted from the casein by the method adopted.

These two experiments have given the same results for the "crude casein" and for the extracted "light-white casein" which were given in the previous method of testing.

It would appear, then, that this offers an alternative method of testing for the unknown factor. Actually, by the time the rats are ready for the test of the unknown substance, they are receiving the same diet in the two ways of testing ("vitamin-free casein," dextrinised rice starch, salt mixture, agar-agar, vitamins B_1 and B_2 as independent preparations or as yeast, and cod-liver oil) whether they began with a shortage of vitamin A as in a vitamin A test or of vitamin B as in a vitamin B test. The former, "vitamin A way," has given us more animals for this work because the tests have necessarily been extended over longer periods (preparatory and test) than in the "vitamin B way," and the rats have apparently exhausted their reserves of the unknown factor. In the "vitamin B way" the rats have a very short preparatory period (10 days) and often a short test period (3–4 weeks), so that the test is often completed before the animals' reserves of the factor are exhausted.

The importance of supplying this factor in the basal diet of all rats used for growth experiments is obvious. It is a simple matter to give "light-white" or a similar sample of casein in the diet of rats preparing for a vitamin B test, for this form of casein is free from either B_1 or B_2. For vitamin A testing, however, a method of removing vitamin A without removing the unknown factor must be adopted. These points will be the subject of a later paper.

Discussion.

Certain food substances have been found to restore growth in rats which had ceased to grow although receiving a diet considered adequate in all factors known to be essential for growth.

It would appear probable that these substances supplied some factor which was missing from the basal diet of the rat, and that those rats which had succeeded in growing to maturity on this diet had reserves of the unknown factor which they had stored from pre-experimental feeding. No satisfactory

extract has yet been obtained from "light-white casein" (B.D.H.), but alcohol and ether extracts of the factor have been prepared from wheat embryo. The fact that it can be so extracted is evidence of its character as a fat-soluble factor.

Another suggestion is that the casein in general use in the vitamin-free diet has, by the process of freeing it from vitamin A, been damaged in some way that has rendered it less digestible or less adequate for growth and that the substances which have restored growth contain "unspoilt" protein. That the active substance can be extracted at all seems to be against this explanation. The fact, too, that many rats grow rapidly on the diet containing the "vitamin-free casein" (Glaxo) shows that it is an adequate protein for some rats at least. Indeed, many rats of both the albino and the black and white strains obviously have been able to digest the protein all their lives. The possibility of racemisation of the protein must be discarded as the activity has been almost completely removed from the protein by hot alcoholic extraction. The fact that the response to the untreated protein is sometimes delayed for many days also seems to be indicative of some actual deficiency rather than of some inability to digest the protein available.

Distinction from vitamin E. At one time it began to appear probable that the factor causing the remarkable resumption of growth would prove to be vitamin E. Its distribution was apparently similar to that of the latter factor; but further work, particularly with wheat-germ oil, makes this at least doubtful. Evans [1928] has demonstrated the growth-promoting power of this factor, and reported that wheat-germ oil in general made a marked difference in growth rate, in the absence of other sources of vitamin E, in a dose of 0·12 g. daily. Three samples which were prepared specially for this laboratory by extraction of wheat germ with light petroleum and concentration *in vacuo* were found to have distinctly less growth-promoting activity than that reported by Evans for vitamin E. One sample prepared by ourselves also showed less activity than was generally experienced by Evans, a dose of 0·16–0·24 g. being necessary to cause a really definite resumption of growth. Moreover, Evans has shown that etiolated pea-shoots are as rich in vitamin E as green ones. Our experience of one species of etiolated shoot (wheat) shows it to be poor in the unknown factor. Further, the liver which we have examined is richer than ox muscle, a relationship which is the reverse of Evans's experience with vitamin E.

The fact that the unknown factor can be destroyed by heat also makes it clear that it is not vitamin E. Moreover, rats suffering from a shortage of it show none of the usual cyclical changes in the reproductive organs as seen by examining the vaginal smears (unpublished work), whereas a shortage of vitamin E has no influence on the oestrous cycle [Evans and Burr, 1927].

There has, moreover, been no indication of a shortage of vitamin E among the animals of the stock colony of this laboratory. (They have had liberal supplies of fresh watercress twice a week, for which we are indebted to

W. Bedford, Esq. of Bere Regis.) Every mating is recorded; each doe is weighed once a week, and oftener during the last week of pregnancy. In the history of some 200 breeding does the occurrence of resorptions has been only about 1 % of the matings which have themselves averaged 4–6 per animal. This factor, then, seems to bear no relation to vitamin E beyond a somewhat similar distribution.

Summary.

Rats often cease to grow even though they are receiving a diet hitherto considered to be satisfactory in calorific value, salt content, and digestibility and also known to contain abundance of all recognised vitamins.

Resumption of growth in these animals was first obtained by the substitution of "light-white casein" (B.D.H.) for the "vitamin-free casein" (Glaxo) generally used in the basal diet.

Other substances which bring about rapid resumption are fresh milk, lettuce, fresh and dried grass, ox muscle, liver and wheat embryo.

Less rapid growth is brought about by watercress and milk which has been simmered for 15 minutes.

Butter and etiolated wheat shoots show very little influence on the growth of these rats.

The power to cause growth resumption is removed from "light-white casein" only with difficulty. Heat can destroy it, at least partially. Cold alcohol, ether, and 2 % acetic acid give inactive extracts and leave unimpaired casein. Boiling alcohol and ether give slightly active extracts and leave definitely impaired casein. The loss may be due to heat.

Boiling alcohol (90 %) and ether give extracts of wheat embryo of definite activity and leave the wheat embryo with only slight activity.

The results here described indicate either the existence of a growth-promoting factor hitherto unrecognised or a biological inadequacy of the protein supplied to the rats in the basal diet. The preparation of active alcohol and ether extracts supports the former view.

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Addendum.

Since this paper was written, we have read that Evans and Burr (*Proc. Soc. Exp. Biol. Med.* 1927, **24**, 740) have demonstrated a deterioration in their 'casein' after long extraction with alcohol and ether.

LXXX. VITAMIN D IN ERGOT OF RYE.

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In the course of experiments carried out with another object it was observed that ergot of rye had a powerful action in promoting calcification of the bones when added to diets which, in themselves, resulted in the development of rickets [Mellanby, Surie and Harrison, 1928]. It was therefore decided to investigate this problem in more detail, to study the properties of the calcifying substance in ergot and to consider its relation to or identity with vitamin D.

Experimental methods.

The animals used in this work were puppies, and the technique was that developed by one of us and described elsewhere [E. Mellanby, 1919, 1921]. It consists essentially in giving young animals of the same litter, from 6 to 8 weeks old, a mixed diet which alone produces rickets. Some are given in addition the substances whose effects on bone development are under investigation and the results on the bones are observed in the test and control animals. Since litters of puppies vary to some extent in their reaction to the basal diet, it is important that in examining the results comparison should be made between the animals in each litter and not between animals in different litters. This caution is also necessary because in the experiments to be described there are variations in the basal diets of different litters so that these diets are sometimes more and sometimes less rickets-producing.

Ergot itself can only be administered to dogs in relatively small quantities owing to its unpleasant taste: even when given in such quantities as from 2 to 5 g. daily the animals often refuse to eat their food, so that it is necessary periodically to omit the substance from the diet. In some experiments the powdered ergot was mixed with bread or meat and administered in pill form in the evening, but even so the food was sometimes refused the following day. In the majority of experiments, therefore, the puppies eating ergot did not grow as well as the other members of the family, a fact which must be taken into account in interpreting nutritional experiments. In spite of these difficulties, however, the antirachitic action of ergot is very evident. When alcoholic and ether extracts of ergot replace the ergot itself the difficulties regarding the food intake are avoided; the animals grow at the same rate as the controls and the results are therefore more satisfactory.

The calcifying action of ergot.

The following sample experiment shows that 2 to 5 g. of ergot added daily to a diet which alone produces rickets assist the calcification processes and tend to prevent rickets.

Exp. 1. Two puppies of one litter were given a rickets-producing basal diet: 1302 received the basal diet only; 1305 was given in addition 2 to 5 g. of powdered ergot.

Age at beginning: 7 weeks.

Duration of experiment: 19 weeks.

Basal diet: 100–160 g. bread, 20 g. lean meat, 20 g. separated milk powder, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 1 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (19 wks.) g.	Gain wt. in 19 wks. g.	Clinical and radiographic diagnosis of bone condition
1302	Basal diet	2400	6985	4585	Bad rickets (Plate II, fig. 1)
1305	Basal diet + 2 to 5 g. of ground ergot	2510	5820	3310	Normal: no rickets (Plate II, fig. 2)

It is evident that the addition of 2 to 5 g. of ergot daily to the basal diet has prevented rickets.

The solubility of the calcifying agent in alcohol.

Exp. 2. 800 g. of ground ergot were extracted twice with 2400 cc. of absolute alcohol. In the first extraction the mixture was boiled for 1 hour and in the second for ½ hour. The extracts were filtered hot and the alcohol was removed from the combined filtrates under reduced pressure, leaving an oily residue.

Four puppies of the same litter were given a rickets-producing basal diet: 1388 received the basal diet only; 1386 was given in addition the alcoholic extract representing 2 to 4 g. of ergot daily; 1387 was given in addition the residue of ergot after extraction with alcohol, equivalent to 2 to 4 g. of ergot daily; 1389 was given in addition 2 to 4 g. of ergot daily.

Age at beginning: 6 weeks.

Duration of experiment: 11 weeks.

Basal diet: 70–80 g. oatmeal, 20–30 g. separated milk powder, 20 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (11 wks.) g.	Gain wt. in 11 wks. g.	Clinical and radiographic diagnosis of bone condition
1386	Basal diet + alc. ext. of 2 to 4 g. ergot	1725	4720	2995	Rickets (much less than in 1387 and 1388)
1387	Basal diet + ergot residue after alc. extraction	1630	4160	2530	Very bad rickets
1388	Basal diet only	1415	3450	2035	Very bad rickets
1389	Basal diet + 2 to 4 g. ergot	1790	3400	1610	Rickets (much less than in 1387 and 1388)

It is evident that most of the calcifying action of the original ergot is present in the alcoholic extract and that the residue after extraction is devoid of any calcifying effect. The fact that oatmeal forms the cereal in this experiment probably accounts largely for the fact that 1386 and 1389 both have some rickets.

The calcifying effect of the alcohol- and ether-soluble fraction after saponification.

Exp. 3. The oily product obtained from ergot by alcoholic extraction as described in Exp. 2, was well shaken with 500 cc. ether, the insoluble resin being ground up several times with ether to ensure the removal of its ether-soluble contents. The ethereal solution was then filtered and the ether distilled off. This oily residue was dissolved in 100 cc. hot alcohol. 15 g. KOH in 15 cc. water were added and the mixture was boiled under a reflux condenser for 1 hour. The solution was then neutralised with dilute HCl. Portions of this mixture equivalent to 4 g., later to 2 g., of the original ergot were added to the diet of 1407.

Two puppies of the same litter were given a rickets-producing diet: 1406 received the basal diet only; 1407 was given in addition the solution from 2 to 4 g. of ergot after saponification.

Age at beginning: 9 weeks.

Duration of experiment: 10 weeks.

Basal diet: 70 g. oatmeal, 20–30 g. separated milk powder, 20–10 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (10 wks.) g.	Gain wt. in 10 wks. g.	Clinical and radiographic diagnosis of bone condition
1406	Basal diet	2435	4460	2025	Very bad rickets
1407	Basal diet + solution from 4 to 2 g. of ergot after saponification	2145	4320	2175	Almost normal

It is evident that the calcifying factor of ergot is soluble in ordinary ether and is stable to the treatment which saponifies the fats.

Extraction of the unsaponifiable fraction. Having found that the calcifying factor in ergot was ether-soluble and not destroyed by saponification, it remained to see whether it was, like vitamin D, an unsaponifiable substance which could be removed by the ordinary methods for separating this substance from the saponified product.

Our earlier experiments appeared at first to negative this supposition, for the unsaponifiable fraction as then prepared had little or no antirachitic action. The saponification of the oily fraction was carried out as described in Exp. 3. The product was then diluted with its own volume of water and extracted three times with approximately 750 cc. of light petroleum (B.P. 60–80°). The light petroleum was evaporated off and the unsaponifiable residue added to the diet of dogs to test its calcifying properties. Since this is the ordinary method of preparing fat-soluble vitamins from cod-liver oil, it was surprising to find little or no evidence that the calcifying substance had been extracted by the light petroleum.

In subsequent experiments the extraction was made by ordinary ether instead of light petroleum and definite evidence of the presence of the calcifying substance in the unsaponifiable fraction was then obtained, although the activity of the extract did not represent the full activity of the original ergot. Two experiments, one of a preventive and the other of a curative nature, are given as examples to illustrate the calcifying action of the unsaponifiable substance obtained from the fat-soluble fraction of ergot.

The calcifying effect of the unsaponifiable fraction extracted from the saponified fats by ether.

Exp. 4 (*preventive experiment*). Two puppies of the same litter were given a rickets-producing diet: 1497 received the basal diet plus unsaponifiable substance from 4 g. ergot; 1501 received the basal diet only.

Age at beginning: 7 weeks.

Duration of experiment: 12 weeks.

Basal diet: 80–150 g. white flour, 20 g. separated milk powder, 20 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (12 wks.) g.	Gain wt. in 12 wks. g.	Clinical and radiographic diagnosis of bone condition
1497	Basal diet + unsapon. subs. from 4 g. ergot	2120	5100	2980	Very little rickets (Plate II, fig. 3)
1501	Basal diet	2650	5820	3170	Very bad rickets (Plate II, fig. 4)

It is clear that the unsaponifiable fraction prepared in this way has prevented in 1497 the very defective calcification seen in 1501.

Exp. 5 (*curative experiment*). Two puppies of the same litter were given a rickets-producing diet. After 10 weeks' feeding, when both had developed very bad rickets, the unsaponifiable substances representing 6 g. of ergot were added daily to the diet of 1517, that of 1519 being continued without alteration.

Age at beginning: 7 weeks.

Duration of experiment: 17 weeks.

Basal diet: 100 to 150 g. bread, 20 g. separated milk powder, 20 to 10 g. lean meat, 10 cc. peanut oil, 2·5 g. dried yeast, 3 cc. orange juice, 1 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (10 wks.) g.	Weight at time of X-ray (17 wks.) g.	Clinical and radiographic diagnosis of bone condition: After 10 wks.	After 17 wks.
1517	Basal diet for 10 wks., then unsap. residue from 6 g. ergot for further 7 wks.	2185	4700	5520	Very bad rickets similar to 1519	Healing rickets
1519	Basal diet for 17 wks.	2095	4550	4700	Very bad rickets similar to 1517	Very bad rickets

In this case, the unsaponifiable residue has brought about healing. Puppy 1517 was active to some extent at the end of the experiment, 1519 was paralysed.

Having obtained evidence that the calcifying action of ergot was due to some substance with properties similar to those of vitamin D, we have assumed that the active substance is vitamin D since, so far as is known, this is the only entity which controls the processes of calcification in the body. The problem then arose as to how vitamin D was formed in ergot. Since ergosterol was first discovered as a constituent of ergot by Tanret [1889], and since ergosterol is now known to be transformed into vitamin D by ultra-violet radiation [Rosenheim and Webster, 1927; Hess and Windaus, 1927], the first suggestion as to the mode of origin of vitamin D in ergot was that it was

produced at some stage in the growth of the infected rye by the direct action of the ultra-violet radiations of sunlight on ergosterol. This mode of origin did not, however, appear very probable because ergot of rye has a dark bluish-black covering and there was doubt as to whether this covering was permeable to the radiations. If it were permeable, then, since ergot contains abundant unactivated ergosterol, exposure of the ergot grains to sunshine ought to increase its calcifying activity. In order to test this point the following experiment was carried out.

The effect of irradiating ergot with sunlight and mercury-vapour light.

Exp. 6. A sample of whole ergot grains was scattered sparsely over a tin dish and exposed to bright summer sunshine for 12 hours. Another sample was placed on a similar dish and exposed to a mercury-vapour lamp at a distance of about 2 ft. for half an hour. Each sample was then ground and extracted with alcohol. The alcohol was distilled off from each under diminished pressure and the residues were administered to two dogs on an otherwise poor calcifying diet. The third dog received the alcoholic extract of 2 g. untreated ergot.

Three puppies of the same litter were given a rickets-producing diet: 1498 received the basal diet plus alcoholic extract of ergot irradiated by sunlight; 1499 received the basal diet plus alcoholic extract of ergot irradiated by mercury-vapour light; 1496 received the basal diet plus alcoholic extract of untreated ergot.

Age at beginning: 7 weeks.

Duration of experiment: 12 weeks.

Basal diet: 80–150 g. white flour, 20 g. separated milk powder, 20 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (12 wks.) g.	Gain wt. in 12 wks. g.	Clinical and radiographic diagnosis of bone condition
1496	Basal diet + untreated ergot (alc. ext. 2 g.)	2430	5300	2870	Rickets similar to 1498
1498	Basal diet + sunlight ergot (alc. ext. 2 g. ergot)	2400	5500	3100	Rickets similar to 1496
1499	Basal diet + Hg-vapour light ergot (alc. ext. 2 g. ergot)	2150	5500	3350	Slight rickets, definitely less than 1496 and 1498

The alcoholic extract of 2 g. only of non-irradiated ergot was given to 1496 so that some degree of rickets should develop, thus allowing the detection of an increase (if any) in the vitamin D after irradiating ergot by sunlight (1498) and by the mercury-vapour lamp (1499). The results of the experiment show (1) that exposure of intact ergot grains to strong sunlight for 12 hours does not increase their calcifying activity and (2) that exposure to the mercury-vapour lamp for half an hour slightly increases the activity. It is evident that the covering of the ergot grains is relatively impermeable even to very abundant ultra-violet radiations. This result raised some doubt as to whether the presence of vitamin D was due to the direct action of sunlight on the ergosterol present in the ergot.

It appeared desirable, then, to decide whether the vitamin D is primarily a product of the sclerotium or whether the rye embryo in which the fungus

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22.6.48	8.3.60	29 NOV 1[illegible]
4.8.48	1.4.1960	
29.7.51	7.7.1960	
25.2.53	26.8.60	
8 DEC 1953	[illegible].2.61	
8.x.55	28.11.61	
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